(54) Title: A METHOD FOR GENERATION OF ANTIBODIES TO CELL SURFACE MOLECULES

(57) Abstract

The present invention relates to a method of generating antibodies directed against cell surface antigens and it uses recombinant insect cells as immunogens. The insect cells have been transfected with coding regions for a molecule containing a cell surface antigen and these antigens are expressed on the surface of the insect cells. Host animals are immunized with these transfected insect cells to generate antibodies directed against the cell surface antigen. Antibody-producing cells from the host animal are used to generate monoclonal antibody-producing hybridoma cells. Sera and hybridoma supernatants can be tested for the presence of antibodies against the surface antigen, using the transfected cells in a screening assay. Specific antibodies that have been generated by the present method are anti-B7 and anti-CD40 antibodies and they can be used to prevent or treat an antibody-mediated or immune system disease in a patient. The anti-B7 antibodies can be used to cause T cell anergy, treat allograft transplant rejection, treat graft versus host disease, and prevent or treat rheumatoid arthritis. An immunosuppressive agent may be co-administered with the antibody. The anti-CD40 antibodies which bind to the CD40 antigen can be used to prevent the growth or differentiation of the B cell. Monoclonal antibodies useful in these methods, and epitopes immunoreactive with such monoclonal antibodies are also presented.

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A METHOD FOR GENERATION OF ANTIBODIES
TO CELL SURFACE MOLECULES

Field of the Invention

The present invention relates to a method of
5   generating antibodies directed against membrane-
associated antigen molecules. More specifically,
the present invention describes methods of using
these membrane-associated antigens for the
immunization of a host animal and for screening
10   antibodies isolated from the host animal. Also,
this invention relates to novel methods of treating
diseases of the immune system. In particular, this
invention relates to methods of preventing or
treating (1) transplant rejection, graft versus host
15   disease, and other immunological conditions arising
from the recognition of specific antigens as
foreign, and (2) antibody-mediated diseases such as
IgE-mediated disease (allergies) and autoimmune
diseases including sytematic lupus erythematosus
20   (SLE), primary biliary cirrhosis (PBC), and
idiopathic thrombocytopenic purpura (ITP).

Background of the Invention
I.   Producing Monoclonal Antibodies.

Monoclonal antibodies have proven to be
25   powerful tools in immunological research. In
general, monoclonal antibodies can be produced using
impure antigens as immunogens, provided that there
is available a screening assay which distinguishes
antibodies directed against the antigen of interest
30   from antibodies directed against other antigens
present in the immunogenic composition. When the
antigen of interest is a cell surface molecule, it
is desirable to use cells or membrane fractions

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containing the molecule of interest as immunogens in order to preserve the conformational constraints provided by a membrane environment.

Immunizing mice with whole cells usually yields
5   a strong immune response which generates antibodies to a large number of different molecules. This broad immune response precludes the use of the immunogen cells in subsequent screening for specific antibody production by hybridoma clones derived from
10   the mouse spleen or lymphocyte cells.

Furthermore, when the antigen of interest is expressed at low density, it is likely that the frequency of mouse B cells specific for the antigen will be relatively low. This low frequency
15   necessitates the screening of large numbers of hybridoma clones to identify a clone which produces antibodies directed against the antigen of interest.

Syngeneic murine fibroblasts expressing human cell surface antigens have been used to immunize
20   mice for specific antibody production (DiSanto et al., 1991). When injected into the appropriate mouse strain, the background antigen proteins present on the fibroblasts should not be immunogenic, so that the immune response should be
25   focused on the xenogeneic recombinant protein. However, this approach requires the construction of specific recombinant cells for each species or strain in which antibody production is desired.

II.   Graft Rejection and the CD28-B7 System.
30   Current strategies for the prevention of graft rejection after transplantation are based on the use of broad acting immunosuppressive agents such as cyclosporin A (CsA), FK506 and corticosteroids.

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These drugs must often be taken over long periods of time and therefore increase the risk of serious infections, nephrotoxicity, and cancer. In addition, not all patients can tolerate high doses

5   of these immunosuppressive agents, often resulting in graft rejection or graft-versus-host disease (GVHD). Optimal prevention of graft rejection should be based on the induction of specific tolerance to the donor tissue. Thus, the ideal drug

10 for prevention of transplant rejection should induce clonal unresponsiveness, or anergy, of donor-reactive T cells, without the need for long-term immunosuppression. Anergy is thought to be the result of intercellular signaling after interaction

15 between the T-cell receptor (TCR) and the peptide-presenting major histocompatibility complex (MHC) antigen in the absence of a "costimulatory" signal. Mueller, D.L. et al., Annu. Rev. Immunol. (1989) 7:445. This costimulatory signal is normally

20 provided by the cell surface of antigen-presenting cells (APCs). Hawrylowicz, C.M. et al., J. Immunol. (1988) 141:4083; and Springer, T.A. et al., Annu. Rev. Immunol. (1987) 5:223.

T cells play an important role during the

25 normal in vivo immune response. They are involved in cytotoxicity, delayed type hypersensitivity, and T cell-dependent antibody production by B cells. Furthermore, T cells can produce a wide variety of lymphokines such as interleukin-2 (IL-2), tumor

30 necrosis factor alpha (TNF-α), lymphotoxin, gamma interferon (IFN-γ), and granulocyte macrophage colony stimulating factor (GM-CSF).

The activation of T cells is the result of ligand-receptor interactions. Under physiological

35 conditions, the TCR/CD3 complex binds to antigenic

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peptides presented by the MHC molecules of APCs.
The TCR/CD3 complex plays two roles in antigen-
induced activation. First, it recognizes a specific
antigen in the context of an antigen-presenting MHC
5   molecule. Then, the recognition event is
transmitted through the plasma membrane by a
signalling mechanism. However, binding of antigen
to the TCR alone is not sufficient for maximum T
cell activation. A number of other accessory
10   molecules on the surface of the T cell are known to
play important roles in adhesion or signalling or
both. For instance, the CD2 molecule on T cells can
bind to LFA-3 on APCs, but it has also been shown
that binding of antibodies to CD2 can augment the
15   signals provided by the TCR/CD3 complex. Other
ligand pairs involved in T cell activation are LFA-
1/ICAM-1, CD4/MHC-class II antigen, VLA-4/VCAM, and,
most importantly, CD28/B7.
        The likely candidate for the costimulatory
20   signal that determines whether TCR-stimulation leads
to full T cell activation or to T cell anergy is
that generated by interaction of CD28 on the T cells
with B7 on APCs. It has been demonstrated *in vitro*
that cross-linking of the CD28 molecule can rescue T
25   cells from becoming anergic. Harding, F.A. et al.,
Nature (1992) 356:607. CD28 is a homodimeric
transmembrane glycoprotein with an apparent
molecular mass of 44 kDa and is a member of the
immunoglobulin superfamily (Aruffo, A. & Seed, B.
30   PNAS (USA) (1987) 84:8573). The CD28 molecule is
expressed on approximately 95% of CD4-positive T
cells and 50% of CD8-positive T cells.
Costimulation of T cells with monoclonal antibody to
the TCR/CD3 complex and CD28 results in greatly
35   enhanced T cell activation. Thompson, C.B. et al.,

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PNAS (USA) (1989) 86:1333-1337; June, C.H. et al.,
J. Immunol. (1989) 143:153-161; and Lindsten, T. et
al., Science (1989) 244:339-343. This effect
apparently involves stabilization of mRNA for
5   several lymphokines, including IL-2, resulting in a
greatly enhanced production of these lymphokines.
June, C.H. et al., supra; and Lindsten, T. et al.,
supra. Furthermore, a CD28-responsive element has
been demonstrated in the enhancer of the IL-2 gene,
10   suggesting that there is also regulation at the
transcriptional level. Fraser, J.D. et al., Science
(1991) 251:313 and Verwey, C.L. et al., J. Biol.
Chem. (1991) 266:14179-14182. Certain models of T
cell activation mediated by CD28 have been reported
15   to be relatively resistant to inhibition with CsA.
June, C.H. et al., Mol. Cell. Biol. (1987) 7:4472-
4481.
        B7 is a monomeric transmembrane glycoprotein
with an apparent molecular mass of 45-65 kDa and is,
20   like CD28, a member of the immunoglobulin
superfamily. Freeman, G.J. et al., J. Immunol.
(1989) 143:2714-2722. Moreover, B7-expressing CHO
cells are able to synergize with TCR stimulation,
resulting in IL-2 secretion and T cell
25   proliferation. Linsley, P.S. et al., J. Exp. Med.
(1991) 137:721-730; and Gimmi, C.D. et al., PNAS
(USA) (1991) 88:6575. B7 also binds to a
recombinant fusion protein of the CTLA-4 molecule.
Linsley, P.S. et al., J. Exp. Med. (1991) 174:561-
30   569. CTLA-4, too, is a member of the immunoglobulin
superfamily, and the cytoplasmic regions of CTLA-4
and CD28 show significant homology. Harper, K. et
al., J. Immunol. (1991) 147:1037. The B7 molecule
is expressed on activated B cells (Freeman, G.J. et
35   al., supra), monocytes stimulated with IFN-γ

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(Freedman, A.S. et al., Cell. Immunol. (1991)
137:429-437), and isolated peripheral blood
dendritic cells (Young, J.W. et al., J. Clin.
Invest. (1992) 90:229-237). Immunohistochemical
5   studies have shown that the B7 molecule is also
constitutively expressed *in vivo* on dendritic cells
in both lymphoid and non-lymphoid tissue.
Vandenberghe, P. et al., "International Immunology"
(1993).

10   *In vivo*, the B7 antigen is involved in T cell
activation during transplant rejection. Lenschow
and co-workers have used a soluble fusion protein of
human CTLA-4 and the immunoglobulin G1 Fc region
(CTLA4Ig), which strongly binds to both mouse and
15 human B7, to prevent rejection of human pancreatic
islets after transplantation in mice (Lenschow, D.J.
et al., Science (1992) 257:789-792). Here CTLA4Ig
blocks rejection of a xenoantigen (an antigen
foreign to the species from which the T cell is
20 derived).

        Molecules that interfere with the interaction
between the B7 and CD28 antigens are known in the
art. The soluble CTLA4-Ig fusion protein is known
to partially block this interaction. Linsley, P.S.
25 et al., J. Exp. Med. (1991) 74:561. Anti-CD28
antibodies are also known to block this interaction.
Furthermore, anti-B7 antibodies are known in the
art. Yokochi, T. et al., J. Immunol. (1982)
128:823; Freedman, A.S. et al. J. Immunol. (1987)
30 139:3260; Valle, A. et al. Immunol (1990) 69:531.

III. B-Cell Activation and the CD40 Antigen-Ligand
System.

        B cells play an important role during the
normal *in vivo* immune response. A foreign antigen

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will bind to surface immunoglobulins on specific B
cells, triggering a chain of events including
endocytosis, processing, presentation of processed
peptides on MHC-class II molecules, and up-
5   regulation of the B7 antigen on the B-cell surface.
A specific T cell then binds to the B cell via T-
cell receptor (TCR) recognition of processed antigen
presented on the MHC-class II molecule. Stimulation
through the TCR begins to activate the T cell and
10   initiates T-cell cytokine production. Interaction
between the CD28 antigen on T cells and the B7
antigen on B cells can provide a second signal
further activating the T cell, resulting in high
level cytokine secretion. Additionally, the CD40
15   ligand, which is not expressed on resting human T
cells, is up-regulated on the T-cell surface when
the above-mentioned signals are received. The B
cell is then stimulated by the CD40 ligand through
the CD40 antigen on the B-cell surface, and also by
20   soluble cytokines, causing the B cell to mature into
a plasma cell secreting high levels of soluble
immunoglobulin.

   A few years ago, Zubler et al., J. Immunol.
(1985) 134:3662, observed that a mutant subclone of
25   the mouse thymoma EL-4 line, known as EL4B5, could
strongly stimulate B cells of both murine and human
origin to proliferate and differentiate into
immunoglobulin-secreting plasma cells in vitro.
This activation was found to be antigen-independent
30   and not MHC restricted. For optimal stimulation of
human B cells, the presence of supernatant from
activated human T cells was needed, but a B-cell
response also occurred when EL4B5 cells were
preactivated with phorbol-12-myristate 13-acetate
35   (PMA) or IL-1. Zubler et al., Immunological Reviews

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(1987) 99:281; and Zhang et al., J. Immunol. (1990)
144:2955. B-cell activation in this culture system
is efficient -- limiting dilution experiments have
shown that the majority of human B cells can be
5   activated to proliferate and differentiate into
antibody-secreting cells. Wen et al. Eur. J.
Immunol. (1987) 17:887.

The mechanism by which these mutant EL-4 cells
activate both murine and human B cells has not been
10   elucidated previously. It is, however, clear that
cell-cell contact is required for EL4B5-induced B-
cell activation. First, B cells do not proliferate
in the presence of supernatant from PMA-stimulated
EL4B5 cells. Zubler et al. (1985) supra. Second, B
15   cells do not proliferate when they are separated
from PMA-treated EL4B5 cells by a semipermeable
filter membrane. Zhang et al., supra. Antibodies
against mouse LFA-1, human LFA-1 or human LFA-3 and
antibodies against mouse or human MHC class II
20   molecules do not inhibit EL4B5-induced proliferation
of human or murine B cells. Zubler et al. (1987)
and Zhang et al., supra.

The CD40 antigen is a glycoprotein expressed on
the cell surface of B cells. During B-cell
25   diferentiation the molecule is first expressed on
pre-B cells and then disappears from the cell
surface when the B cell becomes a plasma cell.
Crosslinking of the CD40 molecules with anti-CD40
antibodies mediates a variety of effects on B cells.
30   The CD40 antigen is known to be related to the human
nerve growth factor (NGF) receptor and tumor
necrosis factor-alpha (TNF-α) receptor, suggesting
that CD40 is a receptor for a ligand with important
functions in B-cell activation.
35   A ligand for CD40 has been identified on the

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cell surface of activated T cells. Fenslow et al.,
J. Immunol. (1992) 149:655; Lane et al., Eur. J.
Immunol. (1992) 22:2573; Noelle et al., Proc. Natl.
Acad. Sci. (USA) (1992) 89:6550. cDNA cloning of
5   the CD40 ligand revealed a molecule with
characteristics of a type-II transmembrane
glycoprotein with homology to TNF-α. Armitage et
al., Nature (1992) 357:80 and Spriggs et al., J.
Exp. Med. (1992) 176:1543. The extracellular domain
10   of the CD40 ligand contains two arginine residues
proximal to the transmembrane region, providing a
potential proteolytic cleavage site that could give
rise to a soluble form of the ligand. Expression of
recombinant CD40 ligand has demonstrated that this
15   molecule can stimulate the proliferation of purified
B cells and, in combination with IL-4, mediate the
secretion of IgE. Armitage et al. and Spriggs et
al., supra. It has been reported that abnormalities
in the gene for the CD40 ligand, resulting in the
20   absence of a functional molecule on activated T
cells, is responsible for the occurrence of X-linked
hyper-IgM syndrome, a rare disorder characterized by
the inability of these patients to produce normal
levels of antibody isotypes other than IgM. Allen
25   et al., Science (1993) 259:990; and Korthäuer et
al., Nature (1993) 361:539.

All anti-CD40 antibodies known in the art have
a stimulatory effect on human B cells. Cross-
linking of the CD40 molecule on the B-cell surface
30   using known anti-CD40 antibodies mediates a variety
of effects on B cells. Anti-CD40 monoclonal
antibodies (mAbs) can induce intercellular adhesion,
proliferation and, in combination with certain
cytokines, maturation to antibody secreting cells.
35   For example, known anti-CD40 mAbs have been shown to

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mimic the effects of T helper cells in B-cell
activation. When presented on adherent cells
expressing FcγRII, these antibodies induce B-cell
proliferation. J. Banchereau et al., Science (1989)
5   251:70. Moreover, the known anti-CD40 mAbs can
replace the T helper signal for secretion of IgM,
IgG and IgE in the presence of IL-4. H. Gascan et
al., J. Immunol. (1991) 147:8. Furthermore, known
anti-CD40 mAbs can prevent programmed cell death
10   (apoptosis) of B cells isolated from lymph nodes.
     The present invention provides a method for
generating monoclonal antibody-producing cells,
where the antibodies have binding specificity for a
specific cell-surface molecule; this method
15   overcomes the limitations of the above-described
methods. The invention also provides methods for
the use of anti-B7 antibodies or molecules that bind
to the B7 antigen in conjunction with other
immunosuppressive agents to cause T cell anergy and
20   thereby prevent or treat graft rejection or GVHD.
Furthermore, the present invention also provides new
anti-CD40 antibodies that are (1) capable of
inhibiting the B-cell response and (2) can be used
to prevent or treat antibody-mediated disease.

25   **Summary of the Invention**
     The present invention provides a method of
generating immortalized cell lines capable of
producing a monoclonal antibody specifically
reactive against a selected membrane-associated
30   antigen. In the method, the membrane-associated
antigen is produced on the cell surface of insect
cells, typically via recombinant expression of
antigen coding sequences using baculoviral vectors.
These insect cells are injected into a host animal.

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Cells capable of producing antibodies directed
against the membrane-associated antigen are
recovered; such cells are immortalized for growth
in cell culture. The immortalized cells are
5   screened for production of antibodies specific to
the membrane-associated antigen. The screening is
typically accomplished using a binding assay
employing non-insect cells having the membrane-
associated antigen on the surface. Immortalized
10   cells producing antibodies which bind to the
membrane-associated antigen on the surface of said
non-insect cells are selected.

In one embodiment, DNA sequences encoding the
membrane-associated antigen are inserted operatively
15   into a baculoviral vector suitable for expression of
the antigen in selected insect cells. The vector
carrying the DNA sequences encoding the antigen are
introduced into insect cells, for example, by
transfection with the vector. Insect cells
20   transformed with the vector which produce the
membrane-associated antigen on the cell surface are
selected.

One method of isolating target DNA sequences,
encoding the membrane-associated antigen of
25   interest, is using the polymerase chain reaction
employing either DNA or RNA templates containing the
target DNA sequences. Insect cells useful in the
practice of the present invention can be obtained
from *Spodoptera frugiperda*. Commonly used host
30   animal cells, capable of producing antibody
molecules directed against the membrane associated
antigen, are splenic cells or lymphocytes;
immortalization of the host animal cells is
accomplished by standard procedures. In one
35   embodiment of the present invention, the host animal

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is a mouse.

    In the above described method, a number of non-insect cells, having the membrane-associated antigen on the surface, can be employed, including human

5 cells. Such non-insect cells can be primary or cultured cells. In one embodiment, the non-insect cells can be cultured human lymphocytes, especially transformed B cells.

    In the method of the present invention, useful

10 membrane-associated antigens include the following: human cell surface proteins; antigenic marker proteins for a peripheral blood mononuclear cells, for example, CD40 or B7; and viral antigenic proteins present on the surface of a human cell.

15   The present invention also describes a method of producing sera containing antibodies specific against a selected membrane-associated antigen, where the membrane-associated antigen is present on the cell surface of insect cells, the cells are

20 injected into a host animal, and sera from the animal are screened for the presence of antibodies specific to the membrane-associated antigen. Such screening typically employs a binding assay using non-insect cells having the membrane-associated

25 antigen on the cell surface.

    Sera and hybridoma supernatants can also be tested for the presence of antibodies against a selected membrane-associated antigen using, in a screening assay, recombinant insect cells expressing

30 the antigen.

    The current invention is also based on the discovery that the coadministration of a molecule that binds to the B7 antigen (such as the anti-B7 monoclonal antibody made by the above-described

35 method) and an immunosuppressive agent to a patient

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can induce long-lasting T cell anergy against an
alloantigen (an antigen native to the same species
as the T cell).

Accordingly, this invention provides a method
5   for preventing transplant rejection in a patient,
the method comprising administering to a patient in
need of such treatment therapeutically effective
amounts of (1) a molecule that binds to the B7
antigen and (2) an immunosuppressive agent such as
10   cyclosporin A, FK506, or a corticosteroid, in a
pharmaceutically acceptable excipient. In a
preferred embodiment of this invention, the molecule
that binds to the B7 antigen is an anti-B7 antibody.

Additionally, this invention provides a method
15   for preventing or treating graft versus host disease
(GVHD) in a patient, the method comprising
administering to a patient in need of such treatment
therapeutically effective amounts of (1) a molecule
that binds to the B7 antigen and (2) an
20   immunosuppressive agent such as cyclosporin A,
FK506, or a corticosteroid, in a pharmaceutically
acceptable excipient. In a preferred embodiment of
this invention, the molecule that binds to the B7
antigen is an anti-B7 antibody.

25   Also, this invention provides a method for
preventing or treating rheumatoid arthritis in a
patient, the method comprising administering to a
patient in need of such treatment therapeutically
effective amounts of (1) a molecule that binds to
30   the B7 antigen and (2) an immunosuppressive agent
such as cyclosporin A, FK506, or a corticosteroid,
in a pharmaceutically acceptable excipient.   In a
preferred embodiment of this invention, the molecule
that binds to the B7 antigen is an anti-B7 antibody.
35   In more preferred embodiments of the above

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methods, the anti-B7 antibody is a monoclonal antibody, most preferably the B7-24 monoclonal antibody, made as described herein.

The invention is further based on the discovery
5   of anti-CD40 antibodies, made by the above-described method, that do not stimulate the growth and differentiation of human B cells. In contrast, these antibodies can inhibit human B-cell responses at relatively low concentrations. Accordingly,
10 these antibodies can be used to prevent or treat diseases or conditions that are mediated by antibodies produced by the human B-cell response. These antibodies also recognize novel epitopes on the CD40 antigen useful in modulating the B-cell
15 response.

Accordingly, this invention provides a monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the
20 CD40 antigen prevents the growth or differentiation of the B cell.

Additionally, this invention provides a method for preventing or treating an antibody-mediated disease in a patient, the method comprising
25 administering to a patient in need of such treatment a therapeutically effective amount of a monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen
30 prevents the growth or differentiation of the B cell, in a pharmaceutically acceptable excipient.

Also, this invention provides a method for preventing or treating an IgE-mediated disease such as an allergy in a patient, the method comprising
35 administering to a patient in need of such treatment

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a therapeutically effective amount of a monoclonal
antibody capable of binding to a human CD40 antigen
located on the surface of a human B cell, wherein
the binding of the antibody to the CD40 antigen
5   prevents the growth or differentiation of the B
cell, in a pharmaceutically acceptable excipient.

        Furthermore, this invention provides a method
for preventing or treating an antibody-mediated
autoimmune disease in a patient, the method
10   comprising administering to a patient in need of
such treatment a therapeutically effective amount of
a monoclonal antibody capable of binding to a human
CD40 antigen located on the surface of a human B
cell, wherein the binding of the antibody to the
15   CD40 antigen prevents the growth or differentiation
of the B cell, in a pharmaceutically acceptable
excipient. Particular autoimmune diseases
contemplated for treatment by this method include
sytematic lupus erythematosus (SLE), primary biliary
20   cirrhosis (PBC), and idiopathic thrombocytopenic
purpura (ITP).

        Additionnally, this invention provides a CD40
antigen epitope capable of competing with the
binding of a CD40 antigen to an anti-CD40 monoclonal
25   antibody wherein the binding of that antibody to a
human CD40 antigen located on the surface of a human
B cell prevents the growth or differentiation of the
B cell.

        In more preferred embodiments of the above
30   objects, the anti-CD40 monoclonal antibody is made
by the above-described methods, and is either 5D12,
3A8 or 3C6.

Brief Description of the Figures
        Figure 1A shows a schematic representation of

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the baculoviral transfer vector pAcC8 and the
sequence of the multiple cloning site. Figure 1B
shows a schematic representation of the generation
of Sf9 cells which express human CD40 or B7 antigen
5   according to the method of the present invention.

Figure 2 shows the sequences of polymerase
chain reaction primers used in the preparation of
coding regions for human CD40 and human B7 antigens.
These primers were constructed on the basis of the
10   published complete DNA coding sequences for antigens
B7 and CD40 (Freeman et al., 1989; Stamenkovic et
al., 1989).

Figure 3 shows the results of ELISA assays
examining the reaction of anti-(B7) monoclonal
15   antibody BB-1 with Sf9 cells infected with AcB7
virus and with Sf9 cells expressing human CD26.

Figure 4 the results of ELISA assays examining
the reaction of anti-(CD40) monoclonal antibody S2C6
with Sf9 cells expressing CD40 and Sf9 cells
20   expressing B7.

Figures 5A-C show the results of the
fluorescent cell staining of EBV-transformed B cell
line ARC cells expressing CD40 or B7.

Figures 6A-F show the results of competition
25   assays using fluorescent cell staining of EBV-
transformed B cell line ARC cells expressing CD40
and B7, as well as soluble CD40 and B7 antigens.

Figure 7 shows the inhibition of anti-CD3-
induced, B7-mediated proliferation of T cells by Fab
30   fragments of anti-B7 Mab B7-24.

Figure 8 shows the inhibition of anti-CD3-
induced, B7-mediated proliferation of T cells by
anti-B7 Mabs B7-24 (squares) or BB-1 (triangles).

Figure 9 shows the effect of blocking B7/CD28
35   interaction using (A) MAb B7-24 or (B) MAb BB-1

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during 3-day primary MLC (closed symbols), and 3-day secondary MLC (open symbols).

Figure 10 shows the inhibition of anti-CD3-induced, B7-mediated proliferation of T cells by
5   CsA. Data are the mean ± S.D. of 3 individual experiments using T cells of different donors.

Figure 11 shows the synergistic effects of anti-B7 Mab B7-24 and CsA in blocking allo-antigen-induced CTL activity during secondary MLC. CTL
10 activity of the T cells was analyzed after restimulation for 3 days with the EBV-transformed B cell line ARC in the presence of medium alone; 400 μg/ml CsA; 10 μg/ml Mab B7-24; or 400 μg/ml CsA and 10 μg/ml Mab B7-24.

15 Figure 12A compares the ability of three new and one old anti-CD40 mAbs to co-stimulate anti-IgM induced human B-cell proliferation. Figure 12B repeats the experiment of Figure 5A in the presence of recombinant interleukin-2 (rIL-2).

20 Figure 13 shows the ability of three new anti-CD40 mAbs to inhibit human B-cell proliferation induced by costimulation with immobilized anti-IgM and anti-CD40 mAb 52C6.

Figure 14 shows the effect of three new anti-
25 CD40 mAbs on EL4B5-induced human B-cell proliferation.

Figure 15 shows the effect of soluble CD40 (hCD40.μ) on EL4B5-induced human B-cell proliferation.

30 Figures 16A and 16B show the effect of one new anti-CD40 mAb 5D12 on human T-cell induced immunoglobulin production by human B cells.

Detailed Description of the Invention

The invention described herein draws on

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previously published work and pending patent
applications. By way of example, such work consists
of scientific papers, patents or pending patent
applications. All of these publications and
5   applications, cited previously or below are hereby
incorporated by reference.

I.   Definitions:

     As used herein, the term "membrane-associated
antigen", "cell surface molecule" and "cell surface
10   antigen" all refer to a protein, polypeptide or
peptide, where at least one antigenic portion of the
protein, polypeptide or peptide is exposed on a
surface of a biological membrane and which may have
one or more of the following moieties covalently
15   attached: one or more simple or complex sugar
moieties (as in a glycoprotein), lipid moieties (as
in a lipoprotein), a combination of lipid and sugar
moieties, or other post-translational modifications.
     "Proteins" are typically long chains of amino
20   acid based polymers ("polypeptides"). Proteins may
be composed of one, two or more polypeptide chains
and may further contain some other type of substance
in association with the polypeptide chain(s), such
as carbohydrates. The size of proteins covers a
25   rather wide range from (an arbitrary figure of)
5,000 to several hundred thousand g/mole. The 5,000
figure corresponds to the presence of roughly 40-45
amino acids. Proteins smaller than about 5,000
g/mole are typically referred to as polypeptides or
30   simply peptides (Bohinski).
     As used herein, the term "antibody" refers to
polyclonal antibodies, monoclonal antibodies,
humanized antibodies, single-chain antibodies, and
fragments thereof such as F_{ab}, $F_{(ab')2}$, F_v, and other

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fragments which retain the antigen binding function
of the parent antibody.

As used herein, the term "monoclonal antibody"
refers to an antibody composition having a
5   homogeneous antibody population. The term is not
limited regarding the species or source of the
antibody, nor is it intended to be limited by the
manner in which it is made. The term encompasses
whole immunoglobulins as well as fragments such as
10 F_{ab}, $F_{(ab')2}$, F_v, and others which retain the antigen
binding function of the antibody. Monoclonal
antibodies of any mammalian species can be used in
this invention. In practice, however, the
antibodies will typically be of rat or murine origin
15 because of the availability of rat or murine cell
lines for use in making the required hybrid cell
lines or hybridomas to produce monoclonal
antibodies.

As used herein, the term "humanized antibodies"
20 means that at least a portion of the framework
regions of an immunoglobulin are derived from human
immunoglobulin sequences.

As used herein, the term "single chain
antibodies" refer to antibodies prepared by
25 determining the binding domains (both heavy and
light chains) of a binding antibody, and supplying a
linking moiety which permits preservation of the
binding function. This forms, in essence, a
radically abbreviated antibody, having only that
30 part of the variable domain necessary for binding to
the antigen. Determination and construction of
single chain antibodies are described in U.S. Patent
No. 4,946,778 to Ladner et al.

As used herein, the term "molecule which binds
35 to the B7 antigen" means a molecule which is capable

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of forming a complex with the B7 antigen in an
environment wherein other substances in the same
environment are not complexed to the B7 antigen.
The complex is formed in a manner that blocks the
5   normal signal transduction pathway of B7 through the
CD28 or CTLA4 antigen. Molecules which bind to the
B7 antigen include CD28, CTLA4, CTLA4Ig and anti-B7
antibodies.

The term "CD40 antigen epitope" as used herein
10   refers to a molecule which is capable of immuno-
reactivity with the anti-CD40 monoclonal antibodies
of this invention, excluding the CD40 antigen
itself. CD40 antigen epitopes may comprise pro-
teins, protein fragments, peptides, carbohydrates,
15   lipids, and other molecules, but for the purposes of
the present invention are most commonly proteins,
short oligopeptides, oligopeptide mimics (*i.e.*,
organic compounds which mimic the antibody binding
properties of the CD40 antigen), or combinations
20   thereof. Suitable oligopeptide mimics are
described, *inter alia*, in PCT application US91/
04282.

II. Generating Antibodies to Membrane-Associated
     Antigen Molecules
25   This section describes a method for generating
and selecting antibodies to a cell surface molecule,
using transfected insect cells as immunogen. The
transfected insect cells of the present invention
may also be used in screening assays.
30   According to an one embodiment, the invention
includes a method for producing polyclonal
antibodies to a cell surface antigen. The method
involves the following steps: the immunization
step, which includes (i) selecting and isolating a

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nucleic acid coding sequence which encodes the
antigen of interest, (ii) inserting the coding
sequence into a baculoviral expression vector so as
to obtain efficient expression of the coding
5   sequence, (iii) transfecting the expression vector
into an insect cell line to obtain recombinant
insect cells expressing the selected antigen, and
(iv) immunizing a host animal with the insect cells
expressing the membrane-associated antigen.

10   After immunization, the serum of the host
animal is screened against cells, other than the
insect cells, expressing the antigen of interest.
Alternatively, membrane fractions containing the
antigen of interest, or in some cases, purified
15   recombinantly-produced antigens themselves can be
used to screen the serum. Typically, (a) pre-bleed
serum, (b) the serum of a host animal immunized with
insect cells not expressing the antigen of interest,
and (c) the serum of the host animal immunized with
20   the recombinant insect cells are screened. The
presence of antibodies specifically directed against
the antigen of interest is indicated by negative
reactions with sera (a) and (b), and positive
reactions with serum (c).

25   According to another embodiment, the invention
includes a method for the generation of hybridomas
which produce monoclonal antibodies to a cell
surface protein. The method involves the steps (i)
to (iv) described above. After immunization of the
30   host animal with the recombinant insect cells,
antibody-producing cells are isolated from the
animal. In a preferred embodiment of the invention,
such antibody-producing cells are used to generate
hybridoma cells, which are cloned and used for the
35   production of monoclonal antibodies. Supernatants

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from such hybridoma cells are screened for specific antibody production, for example, using a cell-based screening assay described below.

A.   Isolating Coding Sequences for Membrane
5   Molecules.

The nucleic acid coding sequence for a selected membrane-associated antigen can be isolated based on known amino acid and/or DNA coding sequences for the protein component of the antigen. The coding
10   sequence can be isolated from biological sources by standard procedures (Ausubel, et al.; Maniatis, et al.; Sambrook, et al.) (e.g., hybridization, differential hybridization, cloning and plaque screening, etc.). Alternatively, synthetic
15   oligonucleotide sequences encoding the antigen of interest can be prepared using commercially available automated oligonucleotide synthesizers or may be purchased, for example, from Synthetic Genetics (San Diego, CA). In the case of large
20   coding sequences, the oligonucleotide coding sequence can be synthesized through a series of cloning steps involving a tandem array of multiple oligonucleotide fragments corresponding to the coding sequence (Crea; Yoshio et al.; Eaton et al.).
25   Oligonucleotide coding sequences can be amplified and isolated by standard recombinant procedures (Maniatis et al.; Ausubel et al.) or by polymerase chain reaction (Mullis; Mullis, et al.).

When the sequence of the membrane-associated
30   antigen is known or partially known a specific antigen coding sequence may be isolated. Typically, the antigen coding sequence is isolated from a cDNA library, generated by the insertion of DNA fragments from a selected source into a vector. The cDNA

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library containing DNA fragments from a membrane
antigen-containing source can be constructed using
random fragments cDNA molecules generated from
target RNA molecules. Such a cDNA library is
5   generally constructed using a bacterial system (such
as lambda gt10 (Promega, Madison WI)), but can also
be constructed in a yeast or eukaryotic expression
system using conventional techniques (Ausubel).
      The library is screened (usually by
10   hybridization; Ausubel, et al.; Maniatis, et al.)
for the presence of the membrane-associated antigen
DNA sequence, typically using as a probe an
oligonucleotide having a known or consensus sequence
hybridizable with the antigen coding region. The
15   probe can carrying a number of detection moieties
including radioisotopes, biotin and digoxigenin.
Alternatively, when a nucleic acid probe sequence is
not available, screening for clones carrying the
coding region of interest can be carried out using,
20   for example, immunoscreening (using "PROTOCLONE"
lambda gt11 system, Promega; Young, et al.; Huynh,
et al.).
      Coding regions are isolated from recombinant
isolates giving positive signals (either by
25   hybridization or immunological screening).
Typically, DNA fragments containing the coding
regions are isolated by restriction digestion
followed by size fractionation and fragment
purification. Such nucleic acid coding regions may
30   then be processed for insertion into a baculoviral
transfer vector, such as the vector pAcC8 (Figure
1A), as described in part B, below. Alternative
baculovirus vectors are available including the
vectors pVL1393 (Luckow et al.) and pAC3T3 (Summers
35   et al.).

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Alternately, coding sequences can also be isolated using polymerase chain reaction (PCR) amplification (Mullis; Mullis, et al.). Primers useful for the PCR can be derived from any known

5   nucleic acid sequence. If the exact sequence is not known degenerative primers can be used (Mullis; Mullis, et al.). Typically these primers are two nucleic acid sequences consisting of 8 or more colinear nucleotides, where the two sequences are

10   separate by some defined distance, in order to generate a target sequence (Example 1), and are complementary to opposite strands.

A typical PCR cycle involved the following steps: melting at elevated temperature, followed by

15   annealing, and extension. The reactions are repeated for 25-30 cycles. The PCR products can be digested with restriction enzymes and electrophore- tically resolved using a preparative 1.5% agarose gel. Clone-specific, amplified fragments are

20   typically identified by electrophoretic gel size fractionation. The clone-specific DNA fragments are then recovered from the gel, for example, using the "GENE CLEAN" system (BIO 101, La Jolla CA). If necessary the DNA can be extracted with phenol

25   and/or phenol:chloroform (1:1). Isolated DNA is ethanol precipitated. Following precipitation, the DNA is used for insertion into baculovirus expression vectors.

The generation of Sf9 cells expressing human

30   CD40 or B7 antigens is schematically shown in Figure 1B. As shown, RNA is isolated from a population of Epstein-Barr virus (EBV)-transformed human spleen cells, using standard procedures (Chirgwin, et al.). Total RNA is converted to cDNA using random hexamer

35   priming, according to established methods, and as

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detailed in Example 1.   The DNA molecule encoding
the membrane-associated antigen molecules of
interest is generated by PCR amplification, using
forward and reverse primers having restriction sites
5   for cloning at their 5' termini.   Such cDNA primers,
used in the preparation of coding regions for human
CD40 and human B7 antigens are depicted in Figure 2.
These primers were constructed on the basis of the
published complete DNA coding sequences for antigens
10 B7 and CD40 (Freeman *et al.*, 1989; Stamenkovic *et
al.*, 1989).

    With continuing reference to Figure 1B, the
cDNA is mixed with a forward primer and a reverse
primer, in the presence of a thermostable
15 polymerase, such as polymerase obtained from *Thermus
aquaticus*, a mixture of equimolar deoxynucleotides,
and a buffer system (Example 1).   The mixture is
subjected to amplification in a thermocycler, and
PCR products obtained are subcloned in the
20 polylinker of a baculovirus transfer vector.   One
such vector, pAcC8, is diagrammatically represented
in Figure 1A.   Any of a number of such baculoviral
transfer vectors containing unique restriction
endonuclease sites downstream of the polyhedrin
25 promoter (Miller) can be utilized in the practice of
the present invention: for *Autographica
californica* nuclear polyhedrosis virus (AcNPV) (Wu,
et al.; Matsuura, *et al.*; Takehara, *et al.*) or
Bombyx mori nuclear polyhedrosis virus (pBmNPV)
30 polyhedrin mRNA (Nyunoya, *et al.*; Sekine, *et al.*).
    Before expression in baculovirus, DNA inserts
are typically checked for PCR-induced mutations by
sequencing analysis.

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B.   <u>Inserting an Antigen Coding Sequence into</u>
<u>a Baculoviral Vector</u>.

     Insertion of the membrane-associated antigen
coding region into a baculovirus vector is performed

5   according to established procedures (Ausubel, *et
al.*; Maniatis, *et al.*; Sambrook, *et al.*). Full
length cDNAs encoding human B7 and human CD40 were
generated by PCR using primers with restriction
sites for cloning. The template for PCR

10   amplification was cDNA generated from EBV-
transformed human spleen B cell RNA. Briefly, an
isolated DNA coding region is ligated into the
baculoviral transfer vector or plasmid, such as a
pAcC8 plasmid, so that the membrane-associated

15   coding region is down-stream of the polyhedron
promoter. The polyhedron gene ATG has been mutated
to ATT (Figure 1A) to prevent translational
initiation in recombinant clones that do not contain
a coding sequence with a functional ATG. The

20   resulting plasmid DNA is co-transfected with wild
type baculovirus (AcNPV) into insect cells from
Spodoptera frugiperda (Sf9 cells) to create
recombinant virus particles, via *in vivo*
recombination between the wild type virus and the

25   recombinant vector, carrying the membrane-associated
antigen gene.

     Examples 1-2 describe the isolation of
recombinant baculovirus vectors containing
heterologous segments of DNA: pAcCD40 (encoding a

30   full-length CD40 molecule), pAcCD40-ED/Glu (encoding
the extracellular domain of CD40), pAcB7 (encoding a
full-length B7 molecule) and pCcB7-ED/Glu (encoding
the cellular domain of the B7 molecule).

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C.   Infecting Insect Cells with Baculoviral
      Vectors.

The recombinant viruses described above were
then used to co-infect insect cells (Example 2).
5   These cells then expressed the antigens encoded by
the heterologous DNA inserts.

Sf9 cells (*Spodoptera frugiperda*; Summers, *et
al.*), at a density of 10^6 cells/ml, were infected
with recombinant virus. Recombinant baculovirus-
10   infected Sf9 cells were identified and clonally
purified (Summers *et al.*).

Cells expressing cell surface antigen were
harvested after 48 hours and used for the
immunization of host animals.   For production of
15   secreted recombinant proteins, the cells were
harvested after 72 hours of culture.

The expression of the recombinant molecules on
the cell surface of the Sf9 cells (Example 3) was
tested using an ELISA system (Harlow, *et al.*).
20   Figure 3 shows that the anti-(B7) monoclonal
antibody BB-1 reacted only with Sf9 cells infected
with AcB7 virus, but not with Sf9 cells expressing
human CD26. In contrast, the anti-(CD26) monoclonal
antibody Ta-1 reacted only with the Sf9 cells
25   expressing CD40. Figure 4 shows that the anti-
(CD40) monoclonal antibody S2C6 reacted only with
Sf9 cells expressing CD40, but not with Sf9 cells
expressing B7. These results show the specificity
of the method of the present invention for the
30   production of selected membrane-associated antigens
in the baculovirus system. These results also
indicate that the membrane-associated antigens are
exposed on the surface of the Sf9 cells.

Further, these results suggest that Sf9 cells
35   expressing selected membrane-associated antigens can

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be used to screen sera and hybridoma supernatants for the presence of antibodies reactive against the selected antigen.

D.   <u>Injecting Insect Cells Expressing the
5   Membrane-Associated Antigen into a Host
       Animal</u>.

Appropriate host animals for the production of polyclonal antibodies include, for example, rabbits, goats, sheep, guinea pigs, chimpanzees and dogs.
10 One advantage of the present invention is that immunization adjuvants are generally not required.

Appropriate host animals for use in the production of monoclonal antibodies commonly include rats, hamsters and mice. However, in cases where it
15 is desirable to produce antibodies that are immunologically closer to humans, sources of such antibodies may include higher primates such as chimpanzees. Fusion with a heteromyeloma fusion partner can be used for the generation of monoclonal
20 antibodies (Carroll; Perkins, 1991). Such fusions can be achieved by a number of methods known in the art (Harlow, *et al*.) including exposure of mixed cells to polyethylene glycol and exposure of cells to strong electric field (electrofusion).
25 Hybridomas are selected by growth in selective medium, then are tested for antigen specificity as described below.

For the generation of monoclonal antibodies to CD40 and B7, mice were immunized (Example 5) with
30 the Sf9 cells expressing these molecules on the cell surface. One week after the second immunization, the mice were bled and the sera were analyzed for the presence of specific antibodies using fluorescent cell staining of EBV-transformed B cells

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(Example 3). Figure 5 shows the results of the cell staining which indicate that mice immunized with Sf9 cells expressing CD40 or B7 had a serum titre against EBV-transformed B cell line ARC (American
5   Type Culture Collection (A.T.C.C.), 12301 Parklawn Dr., Rockville MD 20852), which is positive for both CD40 and B7. In contrast, mice which were immunized with control Sf9 cells showed no reactivity with the ARC cells. The results indicate
10 that host animals can be immunized with Sf9 cells expressing a membrane-associated antigen of choice and the immunization results in an immune response including antibodies against the recombinant antigen. The immunization does not result in
15 antibodies cross-reactive with human proteins other than the recombinant human protein cloned in the Sf9 insect cells.

One mouse was given a final booster injection with CD40 expressing Sf9 cells and one with B7
20 expressing Sf9 cells. Three days after the booster injection, the spleens were removed and the splenocytes were fused with SP2/0 murine myeloma cells.

E.   Isolating and Immortalizing Specific
25   Antibody-Producing Lymphocytes.
Antibody-producing lymphocytes for monoclonal antibody production are preferably B-lymphocytes, such as may be isolated from the bone marrow, spleen or lymph nodes of an immune host animal (Harlow, et
30 al.).

Alternatively, B-lymphocytes can be isolated from the peripheral circulation. In this case, blood samples are centrifuged, and are subjected to gradient separation techniques to produce a crude

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peripheral blood lymphocyte (PBL) mixture.
Monocytes and T-lymphocytes are selectively depleted
from this cell mixture according to established
procedures (Mishell). Such remaining cells may be
5 subjected to a selection procedure, such as a
"panning" procedure, in which those cells having
affinity for the antigen are concentrated by
selective capture by an affinity matrix containing
the antigen. In the context of the present
10 invention, such a matrix might comprise a cell which
expresses the membrane-associated antigen.

When B-lymphocytes are isolated from the
circulation as described above, transformation with
a transforming virus, such as Epstein-Barr virus,
15 may be advantageous. Transformed cells
(lymphoblastoids) are dispensed in subculture wells
and maintained in culture for several weeks, prior
to testing for specific antibody production.
Cultures exhibiting such specific antibody
20 production are expanded and fused with species-
appropriate myeloma partner cells using one or more
standard fusion protocols, including polyethylene
glycol, as described above, or electrofusion.
Methods for isolation and immortalization of B-
25 lymphocytes from various sources are known in the
art.

In experiments carried out in support of the
present invention, splenocytes from immunized mice
were fused with SP2/0 murine myeloma cells
30 polyethylene glycol as previously described by de
Boer *et al.* (1988). The hybridoma clones were
processed as described in Example 6.

Table 1 (Example 6) gives a summary of the
fusion data. After the CD40 fusion, only half of
35 the cells were seeded in 480 wells. This resulted

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in 351 wells with hybridoma growth. After the B7
fusion, the cells were distributed in 960 wells and
this fusion yielded 312 wells with hybridoma growth.
Fourteen days after the fusions, supernatants of 12
5   wells were pooled and the pools were tested for the
presence of antibodies reactive with ARC cells.
FACS analysis revealed that 4 pools from the CD40
fusion and 1 pool from the B7 fusion were reactive
with ARC cells. When individual supernatants from
10   the positive pools were retested, 4 wells reactive
with CD40 and 1 well reactive with B7 were
identified. The cells from these positive wells
were cloned by limiting dilution, and, after 3
rounds of cell growth, 4 stable anti-(CD40)
15   hybridoma clones (CD40-3A8, CD40-3C6, CD40-5D12 and
CD40-5 and 1 stable anti-(B7) hybridoma clone (B7-
24) were established. These results indicate the
ability to achieve stable hybridoma clones secreting
monoclonal antibodies directed against a chosen
20   membrane-associated antigen.

A number of methods for screening hybridoma
fusions are available (Harlow, et al.), including:
antibody capture, (i) using labeled antigen, e.g.,
radioactively labelled partially purified or
25   purified antigen, (ii) whole or permeabilized cells,
e.g., Sf9 cells expressing the recombinant antigen;
and antigen capture, (i) antibody/antigen in
solution, (ii) antibody/antigen solid phase.

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F.   Testing the Specificity of the Monoclonal
      Antibodies.

EBV-transformed cells were used for the
screening of the primary hybridoma supernatants and
5   for screening of the subsequent products of the
limiting dilution cloning. Several lines of
evidence presented below suggest that 4 anti-(CD40)
and 1 anti-(B7) monoclonal antibodies have been
generated.

10   First, supernatants from all 5 hybridoma clones
were reactive with ARC cells and other EBV-
transformed B cell lines, but not with T cell lines
HSB (A.T.C.C.) and CEMM (A.T.C.C.).

Second, competition binding experiments were
15   performed using the monoclonal antibodies of the
present invention and soluble forms of the target
antigens (Example 7). Hybridoma supernatants were
pre-incubated with soluble forms of CD40 and B7
(Example 7). Subsequently, the mixtures were added
20   to ARC cells for fluorescent cell staining. The
results of the competition experiment are shown in
Figure 6. The data show that soluble B7, but not
soluble CD40, could block the binding of anti-(B7)
monoclonal antibody B7-24 to ARC cells. Conversely,
25   soluble CD40, but not soluble B7, could block the
binding of anti-(CD40) monoclonal antibody CD40-3A8
to ARC cells. Similar results were obtained with
the other 3 anti-(CD40) monoclonal antibodies.
Furthermore, the effects of soluble CD40 on the
30   anti-(CD40) monoclonal antibodies and the effect of
soluble B7 on the anti-(B7) monoclonal antibody was
concentration dependent. Decreasing the amount of
soluble protein resulted in decreased blocking of
binding of the antibodies to ARC cells.
35   For further analysis, the anti-(CD40) and anti-

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(B7) monoclonal antibodies were tested for their
ability to bind to tonsillar B cells (Example 8).
Table 2 shows that 89-95 percent of freshly isolated
tonsillar B cells stained positive with the four
5 anti-(CD40) monoclonal antibodies. About the same
percentage of cells was positive with anti-(CD40)
monoclonal antibody G28.5 (Clark, *et al.*). Table 3
shows that 12-17 percent of freshly isolated
tonsillar B cells stained positive with anti-(B7)
10 monoclonal antibody B7-24. However, when tonsillar
B cells were cultured for 5 days in the presence of
immobilized anti-(IgM) antibodies and IL-2, the
percentage of cells positive for B7-24 increased up
to about 25 percent.

15   Furthermore, when tonsillar B cells were
stimulated with anti-(IgM) antibodies and IL-2, not
only did the number of B cells positive for B7-24
increase, but there was also a significant increase
in the amount of fluorescent staining per cell,
20 indicating that the expression of B7 was increased
after stimulation.

        The above data indicate that the method of the
present invention provides a way to isolate
monoclonal antibodies which are specifically
25 reactive with membrane-associated antigens. The
monoclonal antibodies obtained by the method of the
present invention can be typed as previously
described (Harlow, *et al.*).

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G. <u>Utility</u>

　　　For the production of monoclonal antibodies it is optimal to immunize mice with purified material. However, purification of membrane antigens requires
5　specialized and complex techniques, and furthermore, extraction from the membrane may alter the structure of the molecule. In addition, solubilization of proteins often decreases their immunogenicity. Therefore, most monoclonal antibodies to cell
10　surface antigens have been obtained after immunization with mice with whole cells or membrane fractions. In many cases, specific lymphocyte subsets have been injected into mice resulting in panels of monoclonal antibodies. These antibodies
15　have been used to isolate and characterize the antigen that they bound. When mice are immunized with whole cells, antibodies to a large number of different molecules are generated. It is therefore difficult to use the same cells for the screening of
20　specific antibody production by the hybridoma clones.

　　　To circumvent the above-mentioned problem, the method of the present invention involves the expression of membrane-associated antigens in insect
25　cells and the use of these insect cells to immunize host animals. Since the introduction of PCR technology (Saiki <i>et al.</i>, 1985; Saiki <i>et al.</i>, 1988; Mullis; Mullis, <i>et al.</i>), it has become relatively straight-forward to clone cDNAs for proteins whose
30　coding nucleic acid coding sequence has been published. One can use PCR primers spanning the complete coding region only, and incorporate restriction sites in these primers to facilitate cloning into expression vectors.

35　　　It has been shown that human intracellular,

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secreted and transmembrane proteins can be expressed
at high levels in insect cells when expressed in the
cells under the regulation of the non-essential
baculovirus gene for the polyhedrin protein (Webb *et
al.*, 1989; reviewed by Luckow, 1990). Experiments
performed in support of the present invention have
shown that only 2 injections with 5×10^6 Sf9 cells
expressing human CD40 or human B7 gave good serum
titres against these antigens. Furthermore, the
insect cells themselves did not evoke an immune
response cross reactive with human cells. This
enabled the use EBV transformed B cells for the
screening of specific antibody production by the
hybridoma clones, with minimal risk of obtaining
false positives. All of the positive primary wells
obtained by the method of the present invention were
in fact specific for the antigen that was used for
immunization.

Antibodies obtained by the method of the
present invention, directed against membrane-
associated antigens, are advantageous for use as
diagnostic agents for the detection of the membrane-
associated antigen. For example, antibodies
directed against cell-surface marker proteins or
viral proteins protruding from the cell surface.

One diagnostic configuration involves use of
anti-viral antibodies capable of detecting viral
specific antigens. The antigens may be detected,
for example, using an antigen capture assay where
viral antigens present in candidate serum samples
are reacted with an antigen-specific monoclonal or
polyclonal antibody. The antibody is bound to a
solid substrate and the antigen is then detected by
a second, different labelled antibody directed
against the anti-viral antibody.

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The anti-viral antibodies obtained by the method of the invention can be used as a means of enhancing an anti-viral immune response since antibody-virus complexes are typically recognized by

5   macrophages and other effector cells. The anti-bodies can be administered in amounts similar to those used for other therapeutic administrations of antibody. For example, pooled gamma globulin is administered at 0.02-0.1 ml/lb body weight during

10   the early incubation of viral diseases such as rabies, measles and hepatitis B to interfere with viral entry into cells. Thus, antibodies reactive with a membrane-associated viral antigen can be passively administered alone, in a "cocktail" with

15   other anti-viral antibodies, or in conjunction with another anti-viral agent to enhance the immune response and/or the effectiveness of an antiviral drug.

       III. Compositions Using Antibodies

20   This invention contemplates the use of antibodies such as those made by the above-described method. The antibodies of the current invention either (1) bind to a human CD40 antigen on the surface of a human B cell and do not stimulate the

25   growth of differentiation of the B cell or (2) bind to the B7 antigen. These antibodies may, however, be polyclonal antibodies, monoclonal antibodies, humanized antibodies, single-chain antibodies, and fragments thereof. The anti-CD40 antibodies are

30   used alone, while the anti-B7 antibodies may be used in a composition that also contains an immunosuppressive agent.

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A.   <u>Antibody Preparation</u>

Monoclonal antibodies 5D12, 3A8, 3C6, and B7-
24 are prepared as in section II of the detailed
description and Examples 1-7 herein. Other
5   monoclonal antibodies of the invention may be
prepared similarly, or as follows. First,
polyclonal antibodies are raised against the CD40 or
B7antigen. Second, monoclonal antibodies specific
for CD40 or B7 are selected.

10   1.   <u>Polyclonal Sera</u>
Polyclonal sera may be prepared by conventional
methods. In general, a solution containing the CD40
or B7 antigen is first used to immunize a suitable
animal, preferably a mouse, rat, rabbit or goat.
15   Rabbits and goats are preferred for the preparation
of polyclonal sera due to the volume of serum
obtainable, and the availability of labeled
anti-rabbit and anti-goat antibodies. Immunization
is generally performed by mixing or emulsifying the
20   antigen-containing solution in saline, preferably in
an adjuvant such as Freund's complete adjuvant, and
injecting the mixture or emulsion parenterally
(generally subcutaneously or intramuscularly). A
dose of 50-200 μg/injection is typically sufficient.
25   Immunization is generally boosted 2-6 weeks later
with one or more injections of the protein in
saline, preferably using Freund's incomplete
adjuvant. One may alternatively generate antibodies
by in vitro immunization using methods known in the
30   art, which for the purposes of this invention is
considered equivalent to <i>in vivo</i> immunization.
Polyclonal antisera are obtained by bleeding
the immunized animal into a glass or plastic
container, incubating the blood at 25ºC for one

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hour, followed by incubating at 4ºC for 2-18 hours.
The serum is recovered by centrifugation (e.g.,
1,000 x g for 10 minutes). About 20-50 ml per bleed
may be obtained from rabbits.

5   2.   Monoclonal Antibodies
        Monoclonal antibodies are prepared using the
method of Kohler and Milstein, Nature (1975)
256:495-96, or a modification thereof. Typically, a
mouse or rat is immunized as described above.
10 However, rather than bleeding the animal to extract
serum, the spleen (and optionally several large
lymph nodes) are removed and dissociated into single
cells. If desired, the spleen cells may be screened
(after removal of nonspecifically adherent cells) by
15 applying a cell suspension to a plate or well coated
with the protein antigen. B-cells expressing
membrane-bound immunoglobulin specific for the
antigen bind to the plate, and are not rinsed away
with the rest of the suspension. Resulting B-cells,
20 or all dissociated spleen cells, are then induced to
fuse with myeloma cells to form hybridomas, and are
cultured in a selective medium (e.g., hypoxanthine,
aminopterin, thymidine medium, "HAT"). The result-
ing hybridomas are plated by limiting dilution, and
25 are assayed for the production of antibodies which
bind specifically to the desired immunizing cell-
surface antigen (and which do not bind to unrelated
antigens). The selected mAb-secreting hybridomas
are then cultured either in vitro (e.g., in tissue
30 culture bottles or hollow fiber reactors), or in
vivo (as ascites in mice).
        If desired, the antibodies (whether polyclonal
or monoclonal) may be labeled using conventional
techniques. Suitable labels include fluorophores,

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chromophores, radioactive atoms (particularly ^{32}P and
^{125}I), electron-dense reagents, enzymes, and ligands
having specific binding partners. Enzymes are
typically detected by their activity. For example,
5   horseradish peroxidase is usually detected by its
ability to convert 3,3',5,5'-tetramethylbenzidine
(TMB) to a blue pigment, quantifiable with a
spectrophotometer. "Specific binding partner"
refers to a protein capable of binding a ligand
10   molecule with high specificity, as for example in
the case of an antigen and a monoclonal antibody
specific therefor. Other specific binding partners
include biotin and avidin or streptavidin, IgG and
protein A, and the numerous receptor-ligand couples
15   known in the art. It should be understood that the
above description is not meant to categorize the
various labels into distinct classes, as the same
label may serve in several different modes. For
example, ^{125}I may serve as a radioactive label or as
20   an electron-dense reagent. HRP may serve as enzyme
or as antigen for a mAb. Further, one may combine
various labels for desired effect. For example,
mAbs and avidin also require labels in the practice
of this invention: thus, one might label a mAb with
25   biotin, and detect its presence with avidin labeled
with ^{125}I, or with an anti-biotin mAb labeled with
HRP. Other permutations and possibilities will be
readily apparent to those of ordinary skill in the
art, and are considered as equivalents within the
30   scope of the instant invention.

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IV.   Compositions Including Immunosuppressive Agents
           A composition of the current invention
comprises two components which together are
therapeutically effective in preventing or treating
5   graft rejection, GVHD, or rheumatoid arthritis.   The
two components are: (1) a molecule that binds to the
B7 antigen such as MAb B7-24; and (2) an
immunosuppressive agent.   Molecules that bind to the
B7 antigen include CD28, CTLA4, CTLA4Ig and anti-B7
10   antibodies as described in Section III above.
           The anti B7antibodies of the invention (or
other molecules that bind to the B7 antigen) are
given in combination with one or more
immunosuppressive agents.   Immunosuppressive agents
15   are agents that block or inhibit the activation or
proliferation of T cells.   The immunosuppressive
agents according to this invention include
cyclosporin A (CsA), corticosteroids (methotrexate,
prednisolone, dexamethasone), FK506, and rapamycin.
20   Preferably the immunosuppressive agent is
cyclosporin A, FK506 or a corticosteroid, most
preferably cyclosporin A

V.   CD40 Antigen Epitopes
           The CD40 antigen epitopes of this invention are
25   molecules that are immunoreactive with anti-CD40
monoclonal antibodies whose binding to a human CD40
antigen located on the surface of a human B cell
prevents the growth or differentiation of the B
cell.   That is, such epitopes compete with the
30   binding of said antibodies to the CD40 antigen.
Systematic techniques for identifying these epiotpes
are known in the art, as described by H.M. Geysen in
U.S. Patent No. 4,708,871, which is incorporated
herein by reference.   Typically these epitopes are

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short amino acid sequences. These sequences may be embedded in the sequence of longer peptides or proteins, as long as they are accessible.

5   The epitopes of the invention may be prepared by standard peptide synthesis techniques, such as solid-phase synthesis. Alternatively, the sequences of the invention may be incorporated into larger peptides or proteins by recombinant methods. This is most easily accomplished by preparing a DNA

10   cassette which encodes the sequence of interest, and ligating the cassette into DNA encoding the protein to be modified at the appropriate site. The sequence DNA may be synthesized by standard synthetic techniques, or may be excised from the

15   phage pIII gene using the appropriate restriction enzymes.

Epitopes identified herein may be prepared by simple solid-phase techniques. The minimum binding sequence may be determined systematically for each

20   epitope by standard methods, for example, employing the method described by H.M. Geysen, U.S. Pat. No. 4,708,871. Briefly, one may synthesize a set of overlapping oligopeptides derived from the CD40 antigen bound to a solid phase array of pins, with a

25   unique oligopeptide on each pin. The pins are arranged to match the format of a 96-well microtiter plate, permitting one to assay all pins simultaneously, e.g., for binding to an anti-CD40 monoclonal antibody. Using this method, one may

30   readily determine the binding affinity for every possible subset of consecutive amino acids.

Analogs of the invention are also prepared by standard solid-phase methods, and those methods described in PCT application US91/04282.

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VI.   Formulations and Methods of Administration

The antibodies and compositions of this
invention are administered at a concentration that
is therapeutically effective to halt transplant
5   rejection, or prevent or treat (1) GVHD or
rheumatoid arthritis, or (2) antibody-mediated
diseases such as allergies, SLE, PBC and ITP. To
accomplish this goal, the antibodies or compositions
may be formulated using a variety of acceptable
10   excipients known in the art. Typically, the
antibodies or compositions are administered by
injection, either intravenously or
intraperitoneally. Methods to accomplish this
administration are known to those of ordinary skill
15   in the art. It may also be possible to obtain
compositions which may be topically or orally
administered, or which may be capable of
transmission across mucous membranes.

Before administration to patients, formulants
20   may be added to the antibodies. A liquid
formulation is preferred. For example, these
formulants may include oils, polymers, vitamins,
carbohydrates, amino acids, salts, buffers, albumin,
surfactants, or bulking agents. Preferably
25   carbohydrates include sugar or sugar alcohols such
as mono, di, or polysaccharides, or water soluble
glucans. The saccharides or glucans can include
fructose, dextrose, lactose, glucose, mannose,
sorbose, xylose, maltose, sucrose, dextran,
30   pullulan, dextrin, alpha and beta cyclodextrin,
soluble starch, hydroxethyl starch and
carboxymethylcellulose, or mixtures thereof.
Sucrose is most preferred. "Sugar alcohol" is
defined as a C_4 to C_8 hydrocarbon having an -OH group
35   and includes galactitol, inositol, mannitol,

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xylitol, sorbitol, glycerol, and arabitol. Mannitol
is most preferred. These sugars or sugar alcohols
mentioned above may be used individually or in
combination. There is no fixed limit to amount used
5   as long as the sugar or sugar alcohol is soluble in
the aqueous preparation. Preferably, the sugar or
sugar alcohol concentration is between 1.0 w/v% and
7.0 w/v%, more preferable between 2.0 and 6.0 w/v%.
Preferably amino acids include levorotary (L) forms
10   of carnitine, arginine, and betaine; however, other
amino acids may be added. Preferred polymers
include polyvinylpyrrolidone (PVP) with an average
molecular weight between 2,000 and 3,000, or
polyethylene glycol (PEG) with an average molecular
15   weight between 3,000 and 5,000. It is also
preferred to use a buffer in the composition to
minimize pH changes in the solution before
lyophilization or after reconstitution. Most any
physiological buffer may be used, but citrate,
20   phosphate, succinate, and glutamate buffers or
mixtures thereof are preferred. Most preferred is a
citrate buffer. Preferably, the concentration is
from 0.01 to 0.3 molar. Surfactants that can be
added to the formulation are shown in EP Nos.
25   270,799 and 268,110.

Additionally, antibodies can be chemically
modified by covalent conjugation to a polymer to
increase their circulating half-life, for example.
Preferred polymers, and methods to attach them to
30   peptides, are shown in U.S. Patent Nos. 4,766,106;
4,179,337; 4,495,285; and 4,609,546 which are all
hereby incorporated by reference in their
entireties. Preferred polymers are polyoxyethylated
polyols and polyethylene glycol (PEG). PEG is
35   soluble in water at room temperature and has the

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general formula: $R(O-CH_2-CH_2)_nO-R$ where R can be
hydrogen, or a protective group such as an alkyl or
alkanol group.   Preferably, the protective group has
between 1 and 8 carbons, more preferably it is

5   methyl.   The symbol n is a positive integer,
preferably between 1 and 1,000, more preferably
between 2 and 500.   The PEG has a preferred average
molecular weight between 1000 and 40,000, more
preferably between 2000 and 20,000, most preferably

10   between 3,000 and 12,000.   Preferably, PEG has at
least one hydroxy group, more preferably it is a
terminal hydroxy group.   It is this hydroxy group
which is preferably activated to react with a free
amino group on the inhibitor.   However, it will be

15   understood that the type and amount of the reactive
groups may be varied to achieve a covalently
conjugated PEG/antibody of the present invention.

        Water soluble polyoxyethylated polyols are also
useful in the present invention.   They include

20   polyoxyethylated sorbitol, polyoxyethylated glucose,
polyoxyethylated glycerol (POG), etc.   POG is
preferred.   One reason is because the glycerol
backbone of polyoxyethylated glycerol is the same
backbone occurring naturally in, for example,

25   animals and humans in mono-, di-, triglycerides.
Therefore, this branching would not necessarily be
seen as a foreign agent in the body.   The POG has a
preferred molecular weight in the same range as PEG.
The structure for POG is shown in Knauf et al.,

30   1988, J. Bio. Chem. 263:15064-15070, and a
discussion of POG/IL-2 conjugates is found in U.S.
Patent No. 4,766,106, both of which are hereby
incorporated by reference in their entireties.

        Another drug delivery system for increasing

35   circulatory half-life is the liposome.   Methods of

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preparing liposome delivery systems are discussed in
Gabizon et al., Cancer Research (1982) 42:4734;
Cafiso, Biochem Biophys Acta (1981) 649:129; and
Szoka, Ann Rev Biophys Eng (1980) 9:467. Other drug
5   delivery systems are known in the art and are
described in , e.g., Poznansky et al., DRUG DELIVERY
SYSTEMS (R.L. Juliano, ed., Oxford, N.Y. 1980), pp.
253-315; M.L. Poznansky, Pharm Revs (1984) 36:277.
     After the liquid pharmaceutical composition is
10   prepared, it is preferably lyophilized to prevent
degradation and to preserve sterility. Methods for
lyophilizing liquid compositions are known to those
of ordinary skill in the art. Just prior to use,
the composition may be reconstituted with a sterile
15   diluent (Ringer's solution, distilled water, or
sterile saline, for example) which may include
additional ingredients. Upon reconstitution, the
composition is preferably administered to subjects
using those methods that are known to those skilled
20   in the art.
     As stated above, the anti-CD40 antibodies and
compositions of this invention are used to treat
human patients to prevent or treat antibody-mediated
diseases such as allergies, SLE, PBC and ITP. The
25   anti-B7 antibodies are preferably used to prevent or
treat transplant rejection, GVHD or rheumatoid
arthritis. The preferred route of administration
for these antibodies is parenteral. In parenteral
administration, the compositions of this invention
30   will be formulated in a unit dosage injectable form
such as a solution, suspension or emulsion, in
association with a pharmaceutically acceptable
parenteral vehicle. Such vehicles are inherently
nontoxic and nontherapeutic. Examples of such
35   vehicles are saline, Ringer's solution, dextrose

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solution, and Hanks' solution. Nonaqueous vehicles
such as fixed oils and ethyl oleate may also be
used. A preferred vehicle is 5% dextrose in saline.
The vehicle may contain minor amounts of additives
5   such as substances that enhance isotonicity and
chemical stability, including buffers and
preservatives.

The dosage and mode of administration will
depend on the individual. Generally, the
10 compositions are administered so that antibodies are
given at a dose between 1 μg/kg and 20 mg/kg, more
preferably between 20 μg/kg and 10 mg/kg, most
preferably between 1 and 7 mg/kg. Preferably, it is
given as a bolus dose, to increase circulating
15 levels by 10-20 fold and for 4-6 hours after the
bolus dose. Continuous infusion may also be used
after the bolus dose. If so, the antibodies may be
infused at a dose between 5 and 20 μg/kg/minute,
more preferably between 7 and 15 μg/kg/minute.
20   The following examples illustrate, but in no
way are intended to limit, the present invention.

Materials and Methods

Iscove's modification of Dulbecco's Eagle
medium (IMDM) and foetal bovine serum were obtained
25 from JR Biosciences (Lenexa, KS); penicillin and
streptomycin were obtained from Irvine (Santa Ana,
CA); and polyethylene glycol (mol. wt. 1500) was
obtained from Boehringer Mannheim (Indianapolis,
IN).
30   Culture Media. SP2/0 murine myeloma cells,
hybridoma cells, purified T cells, EBV-transformed B
cells and cell lines were cultured in IMDM
supplemented with streptomycin (200 μg/ml),
penicillin (200 U/ml) and 10% heat inactivated

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foetal bovine serum (complete IMDM). The Sf9 insect
cells were cultured in shaker flasks agitated (125-
150 rpm) in medium described by Maiorella et al.
(1989) supplemented with 0.5% foetal bovine serum.

5   3T6-FcγRII cells were cultured in medium consisting
of 50% Dulbecco's modified Eagle's medium and 50%
HAM-F10 medium, supplemented with aminopterin (0.2
μg./ml), thymidine (5 μg/ml), xanthine (10 μg/ml),
hypoxanthine (15 μg/ml), mycophenolic acid (20

10 μg/ml) deoxycytidine (2.3 μg/ml), and 10% heat-
inactivated fetal bovine serum (complete DME/HAM-
F10). 3T6-FcγRII/B7 cells were cultured in complete
DME/HAM-F10 medium containing 400 μg/ml G418
(Gibco).

15   Cells and Cell Lines. Peripheral blood
mononuclear cells were isolated from heparinized
blood (obtained from healthy volunteers) by Ficoll-
Hypaque density centrifugation. T cells were
enriched by depleting monocytes and B cells using

20 Lymphokwik (Lambda, California) (1 λ). The EBV-
transformed B cell line ARC and the P815 cell line,
a NK-resistant murine mastocytoma cell line that
expresses FcγRII and FcγRIII (Ra, C. et al., Nature
(1989) 341:752), were obtained from the ATCC

25 (Rockville, MD). 3T6-FcγRII, the mouse fibroblast
cell line expressing CD32, the human FcγRII high
responder allele, as described by Warmerdam, P.A.M.
et al., J. Exp. Med. (1990) 172:19, was kindly
provided by Dr. J. van de Winkel (University

30 Hospital, Utrecht, The Netherlands). The mutant
mouse thymoma EL-4 subclone EL4B5 was a gift of Dr.
R.H. Zubler, Hôpital Cantonal Universitaire, Geneva.
Mouse 3T6 transfectant cells expressing hybrid
molecules of the HR (high responder) allelic form of

35 human FcγRIIa were a gift of Dr. P.A.M. Warmerdam,

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Department of Experimental Immunology, University
Hospital Utrecht, Utrecht, The Netherlands.
Warmerdam et al., J. Immunol. (1991) 147:1338. Both
cell lines were cultured in Iscove's Modified
5   Dulbecco's Medium (IMDM), supplemented with
gentamycin (80 μg/ml) and 10% heat-inactivated fetal
calf serum (FCS) (Hyclone, Logan, Utah). To avoid
possible loss of B cell activating capacity, every 4
to 8 weeks a new batch of EL4B5 cells was thawed.
10 The cell lines were periodically tested for
mycoplasma contamination by the use of a ^{3}H-labelled
DNA probe for mycoplasma ribosomal RNA (GenProbe,
San Diego, CA) and were free of mycoplasma during
the course of the experiments.
15   Human B Lymphocytes. B lymphocytes were
isolated from tonsils obtained from children
undergoing tonsillectomies, essentially as described
in De Groot et al., Lymphokine Research (1990)
9:321. Briefly, the tissue was dispersed with
20 scalpel blades, phagocytic and NK cells were
depleted by treatment with 5 mM L-leucine methyl
ester and T cells were removed by one cycle of
rosetting with sheep erythrocytes (SRBC) treated
with 2-aminoethyl isothiouronium bromide. The
25 purity of the resulting B lymphocyte preparations
was checked by indirect immunofluorescent labelling
with anti-(CD20) mAb B1 (Coulter Clone, Hialeah, FA)
or anti-(CD3) mAb OKT3 (Ortho, Raritan, NJ) and a
FITC-conjugated F(ab')$_2$ fragment of rabbit anti-
30 (mouse Ig) (Zymed, San Francisco, CA), and FACS
analysis. The B cell preparations contained (mean ±
SD of 6 isolations): 95 ± 4% CD20-positive cells
and 2 ± 1% CD3-positive cells.
        Antibodies. Anti-(human B7) monoclonal
35 antibody BB-1 (Yokochi et al., 1982) was obtained

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from Dr. E.A. Clark (University of Washington,
Seattle, WA) and was used as purified antibody.
Anti-(human CD40) monoclonal antibody G27.5 (Clark
et al., 1986) was obtained from Dr. J.A. Ledbetter
5   (Oncogen Corporation, Seattle, WA) and was used as
purified antibody. Anti-(CD40) monoclonal antibody
S2C6 (Paulie et al., 1985) was obtained from Dr. S.
Paulie (University of Stockholm, Stockholm, Sweden)
and was used as purified antibody. Anti-(human
10 CD26) monoclonal antibody Ta-1 and anti-(CD20)
monoclonal antibody B1 were obtained from Coulter
(Hialeah, FL). Anti-(CD3) monoclonal antibody OKT3
was obtained from Ortho (Raritan, NJ), and the anti-
(LeuM3) monoclonal antibody was obtained from
15 Becton-Dickinson (San Jose, CA). Anti-(IgM)
antibodies coupled to beads (Immunobeads) were
obtained from Bio-Rad (Richmond, CA).

        Anti-B7 Mab B7-24 (IgG2a, x), and anti-CD40
mAbs 5D12, 3C6 and 3A8 were obtained as described
20 in Section II above, and used as purified
antibodies. Anti-CD3 Mab CLB-T3/4.1 (IgG1, x) was
used as diluted tissue culture supernatant and was
kindly supplied by Dr. L. Aarden (Central
Laboratory of the Red Cross Blood Transfusion
25 Service, Amsterdam, The Netherlands). Anti-CD3 Mab
UCHT1 (IgG, x) was used as purified antibody and as
a gift of Dr. P. Beverley (Imperial Research Cancer
Fund, London, UK). Anti-CD72 Mab WL225 (IgG2a, x)
was used as purified antibody and was a gift of Dr.
30 K. Thielemans (Vrije Universiteit Brussel, Belgium).
The anti-ICAM-1 Mab 84H10 was used as diluted
ascites fluid. Anti-CD40 mAb S2C6 was a gift of Dr.
S. Paulie (University of Stockholm, Sweden). Paulie
et al., J. Immunol. (1989) 142:590. Anti-CD40 mAb
35 G28.5 was donated by Dr. J.A. Ledbetter (Oncogen

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Corporation, Seattle, WA, USA). Clark et al., PNAS
(USA) (1986) 83:4494. Control antibodies were:
anti-(β-glucocerebrosidase) mAb 8E4 (IgG1),
Barneveld et al., Eur. J. Biochem. (1983) 134:585,
5   and myeloma immunoglobulins MOPC-21 (IgG1) and MOPC-
141 (IgG2b) (Sigma, St. Louis, MO). All mAb were
used as purified antibody preparations. hCD40.Hμ
fusion protein was a gift of Dr. P. Lane (Basel
Institute for Immunology, Basel, Switzerland) and
10   was used as a 5x concentrated supernatant of
transfected J558L cells. Lane et al., Eur. J.
Immunol. (1992) 22:2573.
      The monoclonal antibodies of the present
invention can be labeled, by standard methods, using
15   a number of reporter moieties, including the
following: fluorescent labels (fluorescein (FITC),
R-phycoerythrin, rhodamine (TMRITC), rhodamine 600
(XRITC), "TEXAS RED," and the like, commonly avidin
linked); radioactive moieties (^{125}I and the like);
20   light-emitting (luciferase and the like); enzymatic
(horseradish peroxidase, alkaline phosphatase,
glucose oxidase, β-galactosidase, and the like).
Further, reporter antibodies (antibodies which have
binding specificity for the monoclonal antibodies of
25   the present invention, e.g., goat anti-mouse IgG)
can also use the above-listed labelling moieties.
      Enzymes and Oligonucleotides. E. coli DNA
polymerase I (Klenow fragment) was obtained from
Boehringer Mannheim Biochemicals (BMB)
30   (Indianapolis, IN). T4 DNA ligase and T4 DNA
polymerase were obtained from New England Biolabs
(Beverly, MA); Nitrocellulose filters are obtained
from Schleicher and Schuell (Keene, NH). Synthetic
oligonucleotide linkers and primers were prepared
35   using commercially available automated oligonucleo-

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tide synthesizers. Alternatively, custom designed
synthetic oligonucleotides may be purchased, for
example, from Synthetic Genetics (San Diego, CA).
cDNA synthesis kit and random priming labeling kits
5   are obtained from Boehringer-Mannheim Biochemical
(BMB, Indianapolis, IN). Oligonucleotide sequences
encoding peptides can be either synthesized as
described above. Alternatively, peptides can be
synthesized directly by standard *in vitro* techniques
10   (Applied Biosystems, Foster City CA).

Common manipulations involved in polyclonal and
monoclonal antibody work, including antibody
purification, were performed by standard procedures
(Harlow, *et al.*).

15   Fluorescent Cell Staining (FACS) Assay. Cells
(10^6/sample) were incubated in 10 μl primary antibody
(10 μl/ml in PBS-BSA or HBSS (Hanks' Balanced Salt
Solution, Gibco/BRL) supplemented with 1% BSA and
0.05% sodium azide) for 20 minutes at 4ºC. After 3
20   washes with PBS-BSA or HBSS-BSA, the cells were
incubated in 100 μl FITC-labeled $F_{ab'2}$ fragments of
goat anti-(mouse IgG) antibodies (Jackson, West
Grove, PA) for 20 minutes at 4ºC. After 3 washes
with PBS-BSA or HBSS-BSA and 1 wash with PBS, the
25   cells were resuspended in 0.5 ml PBS. Analyses were
performed with a FACSSCAN V (Becton Dickinson, San
Jose, CA).

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B7-Mediated T Cell Proliferation Assay.

Purified T cells were cultured with 3T6 fibroblasts transfected with the FcγRIIa high responder allele and the B7 molecule (de Boer, M. et al., Eur. J.

5   Immunol. (1992) 22:3071-3075). Proliferation was measured by ^{3}H-thymidine incorporation. Briefly, 4 x 10^4 T cells were cultured with 10^4 irradiated (2500 rads) 3T6-FcγRII/B7 cells in 96-well flat-bottom tissue culture plates in 200 μl/well complete IMDM

10   with or without anti-CD3 Mab CLB-T3/4.1. During the last 16 hours of a 72 hour culture period, the cells were pulsed with 1 μCi/well ^{3}H-thymidine. Proliferation of T cells is expressed as the mean cpm of triplicate wells.

15   Cytotoxic T Cell Assay. Purified T cells were cultured with 3T6 fibroblasts transfected with the FcγRIIa high responder allele and the B7 molecule. Briefly, 10^6 T cells were cultured with 0.2 x 10^6 irradiated (2500 rads) 3T6-FcγRII/B7 cells in 24-

20   well flat-bottom tissue culture plates in 1 ml/well complete IMDM in the presence of anti-CD3 Mab UCHT1 for 3-4 days. The cytotoxic activity of the lymphocytes was analyzed in an anti-CD3-redirected cytotoxicity assay as described below.

25   Mixed Lymphocyte Culture (MLC) Assay. Proliferation of purified T cells was measured in mixed lymphocyte cultures (MLC) using the EBV-transformed B cell line ARC as stimulator cells. 5 x 10^4 T cells were cultured with 5 x 10^4 irradiated

30   (5000 rads) stimulator cells in 96-well round-bottom tissue culture plates (Corning) in 200 μl/well complete IMDM medium. During the last 16 hours of a 72 hour culture period, the cells were pulsed with 1 μCi/well ^{3}H-thymidine. Proliferation of T cells is

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expressed as the mean cpm of triplicate wells. For
secondary MLCs, cells were stimulated as described
above for primary MLC. The T cell blasts for
secondary MLC were generated in 5- to 7-day primary
5   MLC, with subsequent culture in the absence of the
stimulator cells for 2-4 days. The cytotoxic
activity of T cells generated in primary or
secondary MLC was analyzed in an anti-CD3-redirected
cytotoxicity assay using the mouse P815 cells as
10   described below. Alternatively, the EBV-transformed
B cells used to induce the CTL activity served as
target cells.

     Cytotoxicity Assay. CTL activity was
determined in a 4 hour target cell lysis assay using
15   P815 murine mastocytoma cells or ARC EBV-transformed
B cells as targets. In the case of the P815 target
cells the CTLs were bridged non-specifically to the
target cells using the anti-CD3 Mab OKT3 at 2 μg/ml.
When the ARC cells were used as target cells, only
20   the alloantigen-specific CTLs participate in the
killing process. 10^6 target cells were incubated
with 200 μCi of ^{51}Cr-sodium chromate (Amsersham
International) for one hour and subsequently washed.
The CTL assays were performed in 96-well V-bottom
25   microtiter plates using 5000 ^{51}Cr-labelled target
cells with different amounts of effector cells in a
total volume of 200 μl/well. Four wells were filled
with 5 x 10^3 target cells in 200 μl medium alone, and
four wells with 5 x 10^3 target cells in 100 μl medium
30   and 100 μl saponin (for evaluation of spontaneous
and maximal release, respectively). In the case of
the P815 cells, three wells were filled with
effector cells and target cells in the absence of
anti-CD3 Mab (to determine the background
35   experimental lysis). Three other wells also

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contained the anti-CD3 Mab at 2 μg/ml in order to
determine the total lysis in the presence of anti-
CD3. The plates were centrifuged for 10 minutes at
200 x g and incubated for four hours at 37ºC.

5   Afterwards, 100 μl of the supernatant of each well
was counted in a gamma counter. Results are
expressed as percentage of anti-CD3-dependent
specific release with the P815 target cells, or as a
percentage of alloantigen-specific release with the

10   ARC target cells.

B-Cell Proliferation Assay. B cells (4 x 10^4
per well) were cultured in 200 μl IMDM supplemented
with 10% fetal calf serum in flat bottom 96-well
microtiter plates. B cells were stimulated by

15   addition of immobilized anti-(IgM) antibodies
(Immunobeads; 5 μg/ml; BioRad, Richmond, CA). Where
indicated 100 U/ml recombinant IL-2 was added.
Varying concentrations of mAbs were added at the
onset of the microcultures and proliferation was

20   assessed at day 3 by measurement of the
incorporation of [^{3}H]-thymidine after 18 hour
pulsing.

Banchereau-Like B-Cell Proliferation Assay.
For testing the ability of anti-CD40 mAbs to

25   stimulate B-cell proliferation in a culture system
analogous to that described by Banchereau et al.,
Science (1989) 251:70, mouse 3T6 transfectant cells
expressing the HR allellic form of human FcγRII were
used. B cells (2 x 10^4 per well) were cultured in

30   flat-bottom microwells in the presence of 1 x 10^4
transfectant cells (irradiated with 5000 Rad) in 200
μl IMDM supplemented with 10% fetal calf serum and
100 U/ml recombinant IL-4. Before addition of the B
cells, the 3T6 cells were allowed to adhere to the

35   culture plastic for at least 5 hours. Anti-CD40

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mAbs were added at concentrations varying from 15
ng/ml to 2000 ng/ml and proliferation of B cells was
assessed by measurement of thymidine incorporation
at day 7, upon 18 hour pulsing with [^{3}H]-thymidine.

5   B-Cell Activation Assay with EL4B5 Cells.   B
cells (1000 per well) were cultured together with
irradiated (5000 Rad) EL4B5 cells (5 x 10^4 per well)
in flat bottom microtiter plates in 200 μl IMDM
supplemented with 10% heat-inactivated fetal calf

10   serum, 5 ng/ml phorbol-12-myristate 13-acetate
(Sigma) and 5% human T-cell supernatant. MAbs were
added at varying concentrations at the onset of the
cultures and thymidine incorporation was assessed at
day 6 after 18 hour pulsing with [^{3}H]-thymidine. For

15   the preparation of T-cell supernatant, purified T
cells were cultured at a density of 10^6/ml for 36
hours in the presence of 1 μg/ml PHA and 10 ng/ml
PMA. Wen et al., supra. T-cell supernatant was
obtained by centrifugation of the cells and stored

20   at -20ºC. The effectiveness of T-cell supernatants
in enhancing proliferation of human B cells in
EL4B5-B cell cultures was tested and the most
effective supernatants were pooled and used in the
experiments.

25   Human T Cell Helper Assay for Antibody
Production by B Cells. 96-well tissue culture
plates were coated with a 1:500 dilution of ascites
fluid of anti-CD3 mAb CLB-T3/3 (CLB, Amsterdam, The
Netherlands). As indicated costimulatory mAbs were

30   added: anti CD2 mAbs CLB-T11.1/1 and CLB-T11.2/1
(CLB, Amsterdam, The Netherlands), both ascites
1:1000 and anti-CD28 mAb CLB-28/1 (CLB, Amsterdam,
The Netherlands). Subsequently, tonsillar T cells
(irradiated, 3000 Rad; 10^5 per well), tonsillar B

35   cells (10^4 per well) and rIL-2 (20 U/ml) were added.

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The final volume of each cell culture was 200 μl.
After 8 days, cells were spun down, and cell-free
supernatant was harvested. The concentrations of
human IgM and IgG in (diluted) samples were
5   estimated by ELISA as described below.

    ELISA Assay for Immunoglobulin Quantification.
The concentrations of human IgM and IgG were
estimated by ELISA. 96-well ELISA plates were coated
with 4 μg/ml mouse anti-human IgG mAb MH 16-01 (CLB,
10   Amsterdam, The Netherlands) or with 1.2 μg/ml mouse
anti-human IgM mAb 4102 (Tago, Burlingame, CA) in
0.05 M carbonate buffer (pH = 9.6), by incubation
for 16 h at 4°C. Plates were washed 3 times with
PBS-0.05 % Tween-20 (PBS-Tween) and saturated with
15   BSA for 1 hour. After 2 washes the plates were
incubated for 1 h at 37°C with different dilutions
of the test samples. After 3 washes, bound Ig was
detected by incubation for 1 h at 37°C with 1 μg/ml
peroxidase-labeled mouse anti-human IgG mAb MH 16-01
20   (CLB) or mouse anti-human IgM mAb MH 15-01 (CLB).
Plates were washed 4 times and bound peroxidase
activity was revealed by the addition of o-
phenylenediamine as a substrate. Human standard
serum (H00, CLB) was used to establish a standard
25   curve for each assay.

    Flow Cytofluorometric Assay. ARC cells (10^6
cells/sample) were incubated in 100 μl primary
antibody (10 μg/ml in PBS-BSA or Hanks' balanced
salt solution (HBSS) supplemented with 1% BSA and
30   0.05% sodium azide) for 20 min at 4°C. After 3
washes with PBS-BSA or HBSS-BSA, the cells were
incubated in 100 μl FITC-labeled F(ab')$_2$ fragments of
goat anti-(mouse IgG) antibodies (Jackson, West
Grove, PA) for 20 min at 4°C. After 3 washes with
35   PBS-BSA or HBSS-BSA and 1 wash with PBS, the cells

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were resuspended in 0.5 ml PBS. Analyses were
performed with a FACSCAN V (Becton Dickinson, San
Jose, CA).

     Alternatively, EL4B5 cells were harvested
5 before and at different time points during culture
in medium containing PMA (5ng/ml) and human T-cell
supernatant (5%). Cells were incubated for 30
minutes with 10 μl supernatant of transfected cells
containing hCD40-Hμ diluted in 100 μl Hank's
10 Balanced Salt Solution supplemented with 0.05%
sodium azide (4°C). This was followed by incubation
with FITC-conjugated F(ab')$_2$ fragments of rabbit
anti-(human IgM) (Central Laboratory of the Blood
Transfusion Service, Amsterdam, The Netherlands).
15 As a control, cells were incubated with the FITC-
conjugate only. For analysis a FACScan-4
cytofluorometer (Becton and Dickinson) was used.
Non-vital cells were excluded from analysis by the
use of propidium iodide.

20   **Example 1**
            PCR Cloning of CD40 and B7
     RNA was isolated from a population of EBV-
transformed human spleen cells essentially as
described by Chirgwin *et al.* (1979). In brief, the
25 cells were washed twice with phosphate buffered
saline (PBS) and lysed in 5 M guanidinium
thiocyanate in the presence of 0.7 M 2-
mercaptoethanol. The cell lysate was layered on a
discontinuous CsCl gradient (Chirgwin, *et al.*) and
30 centrifuged for 16 hours at 26,000 rpm in a Beckman
SW28 rotor. The RNA was recovered by dissolving the
pellet in DEPC-treated H$_2$O. The RNA was precipitated
with ethanol once, resuspended in DEPC treated H$_2$O,
and stored at -70°C.

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Total RNA (10 μg/reaction) was converted to cDNA using random hexamer priming in 50 μl reaction buffer containing 500 units LMV-RT (Bethesda Research Laboratories, Bethesda, MD), 5 μM random
5   hexamers (Pharmacia, Piscataway, NJ), 1 mM DTT, dNTP mix (0.5 mM each), 10 mM Tris-HCL pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.1 mg/ml BSA (bovine serum albumin). After incubation at 37°C for 1 hour, the samples were boiled for 3 min and stored at -70°C.
10 The DNA encoding the CD40 and B7 molecules was generated by PCR using primers which contained sequences having homology to known CD40 and B7 sequence, where the primers also encoded restriction sites useful for cloning (Figure 2). These primers
15 were based on the published cDNA coding sequences for B7 and CD40 (Freeman et al., 1989; Stamenkovic et al., 1989). All primers start with a C-G clamp at the 5′ end followed by a restriction site for cloning (shown in bold, Figure 2). The underlined
20 sequences in the backward primers, for the cloning of the soluble forms of B7 and CD40, represents an epitope recognized by a monoclonal antibody used for affinity purification. The numbers in brackets represent the location of the primers relative to
25 the published cDNAs for CD40 and B7.

For PCR amplification, 1 μl of cDNA was mixed 1 μl (10 picomoles) of a forward primer, 1 μl (10 picomoles) of a backward primer, and 47 μl of PCR mix. The PCR mix consisted of 1.25 units Taq
30 polymerase (Perkin-Elmer/Cetus, Norwalk, CT), dNTP mix (0.2 mM each), 10 mM Tris-cHL pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.1 mg/ml BSA. The 50 μl of PCR mixture was overlaid with 70 μl mineral oil and subjected to 25 cycles of amplification in a Perkin-
35 Elmer/Cetus thermocycler (denaturation at 95°C for

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30 sec, primer annealing at 55°C for 30 sec and
extension at 72°C for 1.5 min). PCR products were
obtained after 25 amplification cycles.

    The amplification products were digested with
5   BglII and KpnI (Figure 1B) and isolated by size-
fractionation. Before expression in baculovirus,
the DNA sequence of each fragment was confirmed by
sequencing analysis to prevent the introduction of
PCR-induced mutations. The baculovirus transfer
10   vector pAcC8 was also digested with BglII and KpnI
(Figure 1B).

    The amplified fragments were ligated to the
linear pAcC8 vector (ratio of insert to vector was
3:1). The ligation products were transformed into
15   bacterial strain DH5α (Gibco/BRL, Gaithersburg MD)
and recombinant pAcC8 vectors were selected on the
basis of ampicillin resistance. Recombinant
plasmids were isolated from bacterial clones
(Maniatis, et al.; Ausubel, et al.) and the presence
20   of the insert of interest verified using polymerase
chain reactions (see above). Large scale plasmid
preparation was performed by standard procedures
(Ausubel, et al.; Maniatis, et al.; Sambrook, et
al.).

25   Example 2
          Baculovirus Expression of Human CD40 and B7
    Sequences encoding human CD40 and human B7 were
recombined into the Autographa californica
baculovirus (AcNPV) using the transfer vectors
30   pAcCD40 (encoding the full-length CD40 molecule),
pAcCD40-ED/Glu (encoding the extracellular domain of
CD40), pAcB7 (encoding the full-length B7 molecule)
and pCcB7-ED/Glu (encoding the cellular domain of
the B7 molecule).

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The plasmids were cotransfected with wild-type
baculoviral DNA (2-10 pfu) (AcNPV; Summers *et al.*)
into SF9 (*Spodoptera frugiperda*) cells at a density
of 10^6 cells/ml (Summers *et al.*). Recombinant

5   baculovirus-infected Sf9 cells were identified and
clonally purified (Summers *et al.*).

For cell surface expression of recombinant
proteins the cells were harvested after 48 hours of
culture; for the production of secreted recombinant

10   proteins, the cells were harvested after 72 hours of
culture.

<u>Example 3</u>
<u>Sf9 Cell ELISA</u>

Sf9 insect cells infected with recombinant

15   virus were cultured for 48 hours in 24-well plates.
After removal of the tissue culture medium the
plates were incubated for 45 min at room temperature
(RT) with 0.25 ml of antibody in PBS with 1% BSA
(PBS-BSA). After three washed with PBS-BSA, the

20   plates were incubated for 35 min at RT with 250 μl
of a 1/250 dilution of goat anti-(mouse total Ig)
immunoglobulins conjugated to horseradish peroxidase
(Zymed, South San Francisco, CA) in PBS-BSA.
Unbound peroxidase activity was removed by washing

25   five times with PBS-BSA. Bound peroxidase activity
was revealed by the addition of an assay mixture
prepared by diluting 0.5 ml of 2 mg/ml 3,3',5,5'-
tetramethylbenzidine in ethanol to 10 ml with 10 mM
Na acetate, 10 mM EDTA buffer (pH 5.0) and adding

30   0.03% (v/v) H_2O_2. The reaction was stopped after 10
min by adding 100 μl of 1 M H_2SO_4.

The above-described ELISA assays performed on
live Sf9 cells gave the following results. Figure 3
presents the data for Sf9 cells infected with pAcB7

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and pAcCD26 which were cultured for 48 hours in 24-
well plates. The antibodies used in the ELISA were:
B7-24, anti-(B7) (open bars), Ta-1, anti-(CD26)
(hatched bars) and no primary antibody (gray bars).

5   Figure 4 presents the data for live Sf9 cells
infected with pAcB7 and pAcCd40 which were cultured
for 48 hours in 24-well plates. The antibodies used
in the ELISA were: S2C6, anti-(CD40) (open bars)
and no primary antibody (hatched bars).

10   Example 4
                   Fluorescent Cell Staining
          A.   Fluorescent Cell Staining
          Cells (10^6/sample) were incubated in 10 μl
primary antibody (10 μg/ml in PBS-BSA or HBSS
15   (Hanks' Balanced Salt Solution, Gibco/BRL)
supplemented with 1% BSA and 0.05% sodium azide) for
20 min at 4°C. After 3 washes with PBS-BSA or HBSS-
BSA, the cells were incubated in 100 μl FITC-labeled
Fab '2 fragments of goat anti-(mouse IgG)antibodies
20   (Jackson, West Grove, PA) for 20 min at 4°C. After
3 washes with PBS-BSA or HBSS-BSA and 1 wash with
PBS, the cells were resuspended in 0.5 ml PBS.
Analyses were performed with a FACSSCAN V (Becton
Dickinson, San Jose, CA).

25   General protocols for flow cytometric analysis
and clinical data analysis for flow cytometry are
detailed in Keren *et al*. and Coon *et al*. General
blood cell counting techniques and DNA quantitation
are described by Powers, Keren *et al*., and Coon et
30   *al*.

          The data for fluorescent cell staining of ARC
EBV transformed B cells is presented in Figure 5.
In Figure 5A, the results for staining at 1:100
dilution of serum from a mouse immunized with B7

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expressing Sf9 cells (solid line) or a 1:100
dilution of normal mouse serum (dotted line) are
shown.

In Figure 5B, the results for staining with a
5   1:100 dilution of serum from a mouse immunized with
CD40 expressing Sf9 cells (solid line) or a 1:100
dilution of normal mouse serum (dotted line) are
shown.

In Figure 5C, the results for staining with a
10   1:100 dilution of serum from a mouse immunized with
control Sf9 cells (solid line) or a 1:100 dilution
of normal mouse serum (dotted line) are shown.

B.   Soluble Antigen Competition Assays

ARC EBV-transformed B cells were stained with
15   anti-(B7) and anti-(CD40) monoclonal antibodies in
the presence and absence of soluble B7 and soluble
CD40. The antibodies and the soluble B7, soluble
CD40 or controls were preincubated at RT for 20 min
before addition to the ARC cells.
20   Figure 6A shows the results of staining with
B7-24 (dotted line) or secondary antibody only
(solid line). Figure 6B shows the results of
staining with B7-24 alone (dotted line) or B7-24
preincubated with soluble B7 (solid line). Figure
25   6C shows the results of staining with B7-24 alone
(dotted line) or B7-24 preincubated with soluble
CD40. Figure 6D shows the results of staining with
CD40-3A8 (dotted line) or second antibody alone
(solid line). Figure 6E shows the results of
30   staining with CD407A8 alone (dotted line) or CD403A8
preincubated with soluble B7 (solid line). Figure
6F shows the results of staining with CD403A8 alone
(dotted line) or preincubated with soluble CD40
(solid line).

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Example 5
Host Animal Immunization

Female BALB/c mice were injected
intraperitoneally at day 0 and day 14 with 5×10^6 Sf9
5   cells infected with AcCD40 virus, AcB7 virus or
AcCd3 virus (control virus). At day 21, 100 μl of
serum was obtained to test for the presence of
specific antibodies. After a rest period of at
least two weeks, the mice received a final injection
10 with 5×10^6 cells infected with AcCD40 or AcB7 virus.
Three days after this last injection, the spleen
cells were used for cell fusion.

Example 6
Generation of Hybridoma Clones

15   Splenocytes from immunized BALB/c mice were
fused with SP2/0 murine myeloma cells at a ratio of
10:1 using 50% polyethylene glycol as previously
described by de Boer et al. (1988). The fused cells
were resuspended in complete IMDM medium
20 supplemented with hypoxanthine (0.1 mM), aminopterin
(0.01 mM), thymidine (0.016 mM) and 0.5 ng/ml hIL-6
(Genzyme, Cambridge, MA). The fused cells were then
distributed between the wells of 96-well tissue
culture plates, so that each well contained 1
25 growing hybrid on average.

After 10-14 days the supernatants of the
hybridoma populations were screened for specific
antibody production. For the screening of specific
antibody production by the hybridoma clones, the
30 supernatants of 12 wells were pooled and used for
fluorescent cell staining of EBV-transformed B cells
as described in Example 4. Subsequently, the
supernatants of the positive pools were tested
individually. Positive hybridoma cells were cloned

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three times by limiting dilution in IMDM/FBS
containing 0.5 ng/ml hIL-6. The results of the
analysis are presented in Table 1.

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Table 1

Summary of Fusion Data for
the Generation of Monoclonal
Antibodies to Human CD40 and Human B7

Fusion:	Anti-CD40	Anti-B7
No. of wells seeded after fusion	480[a]	960
No. of wells with hybridoma growth	351	312
No. of positive wells[b]	4	1
Frequency of positive wells[c]	1.15	0.31

[a]Only half of the cells obtained after fusion were analyzed.

[b]As determined by FACS analysis described in Example 4.

[c]The frequency of positive wells is defined as the number of positive wells divided by the total number of wells with hybridoma growth, multiplied by 100.

Example 7
Testing of Tonsillar B Cells

Tonsillar B lymphocytes were isolated from tonsils obtained from children undergoing tonsillectomy as described by deGroot *et al.* (1990). Briefly, the tissue was dispersed with scalpel blades, phagocytic cells and NK cells were depleted by treatment with 5 mM L-leucine methyl ester and T cells were removed by one cycle of rosetting with sheep erythrocytes treated with 2-aminoethyl isothiouronium bromide.

The anti-(CD40) and anti-(B7) monoclonal antibodies, were tested for their ability to bind to tonsillar B cells using the fluorescent cell

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staining assay described above in Example 4.   For
fluorescent stain analysis of cultured tonsillar B
cells, propidium iodine was used to exclude dead
cells.

5   Table 2 shows the results of the above analysis
for the binding of anti-(CD40) monoclonal antibodies
to highly enriched tonsillar B cells.

Table 2

Binding of Anti-(CD40) Monoclonal Antibodies
10   to Highly Enriched Tonsillar B Cells

Antibody	Specificity	% of Positive Cells[a]
OKT3	CD3	2.1
LeuM3	LeuM3	2.5
B1	CD20	88.0
G28.5	CD40	92.1
CD40-5H7	CD40	93.7
CD40-5D12	CD40	95.0
CD40-3C6	CD40	88.9
CD40-3A8	CD40	93.3

20   [a]The percentage of positive tonsillar cells was
measured by fluorescent cell staining as
described in Example 4.

These data show that 89-95 percent of freshly
isolated tonsillar B cells stained positive with the
25   four anti-(CD40) monoclonal antibodies.
The reaction of the tonsillar B cells with

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monoclonal antibody G28.5 (Clark, *et al.*) were
tested in essentially the same manner: about the
same percentage of cells were positive with G28.5 as
with the anti-(CD40) monoclonals of the present
5   invention.

Table 3 shows the results of binding of anti-
(B7) monoclonal antibody B-7-24 to highly enriched
tonsillar B cells as determined by the fluorescent
cell labelling described in Example 4.

10   **Table 3**
Binding of Anti-(B7) Monoclonal Antibody B7-24
to Highly Enriched Tonsillar B Cells

| | | % of Positive Cells[a] | |
Antibody	Specificity	Donor 1	Donor 2
OKT3	CD3	8.0	2.1
B1	CD20	74.0	88.0
B7-24	B7	12.6	16.8

[a]The percentage of positive tonsillar cells was
measured by fluorescent cell staining as
described in Example 4.

20   These data show that 12-17 percent of freshly
isolated tonsillar B cells stained positive with
anti-(B7) monoclonal antibody B7-24.

Example 8
Blocking T Cell Proliferation with Mab B7-24
25   We studied the role of the B7 molecule in T
cell activation using the T cell proliferation and
MLC assays described above. Proliferation in the

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absence of B7-24 Fab fragments ranged from 20,000 to 60,000 cpm. Figure 7 shows that this Mab binds to a functionally important domain of the B7 molecule, since it can completely block anti-CD3-induced, B7-
5   mediated induction of T cell proliferation. Data shown are the ± S.D. of 4 individual experiments using T cells of different donors.

Figure 8 shows the inhibition of anti-CD3-induced, B7-mediated proliferation of T cells by
10   anti-B7 Mabs B7-24 (squares) or BB-1 (triangles). It was therefore surprising to find that Mab B7-24 could not inhibit T cell activation in primary MLC using B7-positive EBV-transformed B cells as stimulator cells. Figure 9 shows the effect of
15   blocking B7/CD28 interaction during primary MLC (closed symbols) and secondary MLC (open symbols). Purified T cells were stimulated with the EBV-transformed B cell line ARC for 3 days in the presence or absence of different concentrations of
20   Mab B7-24 (Figure 9A) or BB-1 (Figure 9B). Proliferation in the absence of antibody ranged from 15,000 to 30,000 in primary MLC and from 20,000 to 60,000 in secondary MLC. Data shown are the mean ± S.D. of 4 individual experiments using T cells from
25   different donors.

From this experiment, it is clear that anti-B7 monoclonal antibodies cannot completely block primary MLCs. Under the same experimental conditions, however, a Mab to CD3 could almost
30   completely block the activation of T cells in the primary MLC. Interestingly, when Mab B7-24 was tested for its inhibitory capacity in secondary mixed lymphocyte cultures using pre-activated T cells, it was able to inhibit the activation of T
35   cells.

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Example 9
Blocking T Cell Proliferation
with Cyclosporin A and/or Mab B7-24

We used the T cell proliferation assay
5   described above to determine whether the
costimulation of T cells with B7 is also resistant
to inhibition with CsA. Figure 10 shows that when T
cells are induced to proliferate with anti-CD3 Mab,
costimulation with B7 can be inhibited in a dose-
10   dependent manner by CsA. However, it was not
possible to completely block the activation of T
cells with CsA concentrations that are not toxic.
When CsA was used to block primary MLC (results not
shown) or secondary MLC (see Table 4), similar
15   results were obtained. These experiments clearly
suggest that B7-CD28/CTLA4 mediated T cell
proliferation is only partially sensitive to the
inhibitory action of CsA. This lack of complete
inhibition when T cells are costimulated with B7 *in*
20   *vitro* could mimic what happens *in vivo* during graft
rejection or acute GVHD despite treatment with CsA.

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Table 4
CsA and Mab B7-24 Synergy in blocking
T-cell Proliferation in Secondary MLC*

B7-24 (μg/ml)	Cyclosporin A concentration						
	$6 \times 10^{-6}M$	$3 \times 10^{-6}M$	$10^{-6}M$	$6 \times 10^{-7}M$	$3 \times 10^{-7}M$	$10^{-7}M$	None
2.5	971	609	601	790	697	1,159	25,209
0.25	553	545	601	788	559	882	20,753
0.025	897	939	1,121	1,592	1,570	2,818	29,364
None	12,687	15,593	23,484	24,589	27,629	33,235	62,598

*Table entries reflect T-cell proliferation in CPM. Proliferation was measured using the T-cell proliferation assay described above. Data shown for one of four experiments.

Table 4 shows that CsA alone or mAb B7-24 alone gave a dose-dependent, but incomplete, inhibition of T cell activation. However, when CsA and B7-24 were combined, T cell activation was completely blocked. Interestingly, addition of 0.025 μg/ml B7-24 gave almost the same amount of blocking as 2.5 μg/ml. Furthermore, in the presence of 0.025 μg/ml B7-24, decreasing the CsA concentration 60-fold still resulted in more than 90% inhibition of T cell activation, being more than the maximal inhibition with the highest CsA concentration alone.

This synergy between Mab B7-24 and CsA was specific for the B7/CD28 interaction, since it was not observed using Mabs to either ICAM-1 or CD72 in the same proliferation assay, as shown in Table 5.

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Table 5

Effects of CsA with B7, ICAM-1

or CD72 Mabs in blocking

T-cell proliferation in a secondary MLC

	Monoclonal Antibody	Concentration (μg/ml)	Cyclosporin A Concentration			
			6×10^{-7} M	3×10^{-7} M	10^{-7} M	None
5	B7-24	2.5	3	3	3	40
		0.25	3	2	3	33
	(α-B7)	0.025	6	6	6	47
	84H10	2.5	76	84	81	115
		0.25	80	80	76	99
10	(α-ICAM-1)	0.025	91	99	96	99
	WL225	2.5	106	107	89	98
		0.25	108	106	91	100
	(α-CD72)	0.025	109	105	93	106

*Table entries reflect T-cell proliferation in CPM. Proliferation was measured using the T-cell

15   proliferation assay described above.

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Example 10
Blocking T Cell Proliferation with Mab B7-24 and
Other
Immunosuppressive Agents

5   The protocol of Example 9 above is used to show the blocking of T cell proliferation using Mab B7-24 in conjunction with other immunosuppressive agents. The proliferation assay protocol in Example 9 is followed substituting (A) FK506, (B) rapamycin, (C)

10   methotrexate, (D) prednisolone, or (E) dexamethasone for CsA.

Example 11
Blocking Cytotoxic T Lymphocyte
Activity with Cyclosporin A and/or Mab B7-24

15   We used the cytotoxicity assay described above to test whether CsA and B7-24 could also cooperate in blocking induction of CTL activity in alloantigen-specific activation of T cells. Purified T cells were stimulated with the EBV-

20   transformed B cell line ARC for 6 days, followed by a 2-day culture period in medium alone. CTL activity of the T cells was analyzed after restimulation for 3 days with the EBV-transformed B cell line ARC in the presence of medium alone; 400

25   μg/ml CsA; 10 μg/ml Mab B7-24; or 400 μg/ml CsA and 10 μg/ml Mab B7-24, as shown in Figure 11. T cells activated by the alloantigen in these cultures were efficiently induced to become cytolytic, since about 50% of the ARC target cells could be lysed in the 4

30   hour assay. This induction of CTL activity in secondary MLC could only be slightly inhibited with 400 ng/ml CsA. Addition of 10 μg/ml Mab B7-24 during the secondary MLC resulted in about 40% inhibition. However, combining CsA and Mab B7-24

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resulted in almost complete blockage of the CTL activation.

Example 12
Blocking Cytotoxic T Lymphocyte Activity with Mab B7-24 and Other Immunosuppressive Agents

5   The protocol of Example11 above is used to show the blocking of cytotoxic T lymphocyte activity using Mab B7-24 in conjunction with other immunosuppressive agents.   The cytotoxicity assay

10   protocol in Example 11 is followed substituting (A) FK506, (B) rapamycin, (C) methotrexate, (D) prednisolone, or (E) dexamethasone for CsA.

Example 13
Combining CsA and Mab B7-24 During Alloantigen-Specific T-Cell Activation

15   We investigated whether T cells stimulated in a primary MLC assay (as described above) with the alloantigen in the presence of CsA and Mab B7-24 could respond to secondary antigen stimulation.

20   Purified T cells were stimulated with the EBV-transformed B cell line ARC for 6 days in the presence or absence of 10 μg/ml Mab B7-24 and 400 μg/ml CsA, followed by a 2 day culture period in medium alone.   CTL activity of the T cells was

25   analyzed after re-stimulation for 3 days with the EBV-transformed B cell line ARC.   The data in Table 6 is that of a representative experiment, showing that 6-day exposure to the alloantigen in the presence of both CsA and Mab B7-24, but not in the

30   presence of CsA or Mab B7-24 alone, resulted in total unresponsiveness for subsequent challenge with the alloantigen in secondary MLC.   This unresponsiveness was not due to lack of viability of

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the cells after the primary MLC, since in a control
experiment the cell population could still be
induced to become cytotoxic after stimulation with
immobilized anti-CD3 Mab or cells expressing a non-
5   related alloantigen (results not shown).

Table 6
Alloantigen-Specific T Cell Tolerance
Induced by the combination of CsA and B7-24

Additions of Culture Medium

10   During Primary MLC*   (% specific release)

	P815	ARC
None	80	49
B7-24 (10 μg/ml)	78	24
CsA (400 ng/ml)	76	37
B7-24 & CsA	0	0

15   B7-24 & CsA

* CTL activity of purified T cells in secondary MLC
with ARC cells as stimulator cells, after primary
MLC as described above. CTL activity measured in T
cell cytotoxicity assay as described above.

20   Example 14
Combining Mab B7-24 and an Immunosuppressive Agent
During Alloantigen-Specific T-Cell Activation
The protocol of Example 13 above is used to
show the ability of Mab B7-24 in conjunction with
25   other immunosuppressive agents to block secondary
antigen stimulation. The CTL assay protocol in
Example 13 is followed substituting (A) FK506, (B)
rapamycin, (C) methotrexate, (D) prednisolone, or
(E) dexamethasone for CsA.

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Example 15
Costimulation of B-Cell Proliferation
Using Anti-CD40 mAbs

Four hybridomas producing monoclonal antibodies
against human CD40 were generated as described in
Examples 1-7. These mAbs were shown to bind to a
similar proportion of tonsillar B cells as anti-CD40
mAb G28.5 does. de Boer et al. J. Immunol. Methods
(1992) 152:15. Three of these monoclonal antiodies
(5D12, 3A8 and 3C6) which were of the IgG2b
subclass, were tested for their ability to deliver
activation signals to human B cells in the B-cell
proliferation assay described above.

Human tonsillar B cells (4×10^4 per well) were
cultured in 200 μl in microwells in the presence of
anti-IgM coupled to Sepharose beads (5 μ/ml) (Figure
12A) or in the presence of anti-IgM plus rIL-2 (100
U/ml) (Figure 12B). Varying concentrations of the
anti-CD40 mAbs S2C6, 5D12, 3C6 or 3A8 were added and
[^{3}H]thymidine incorporation was measured at day 3
after 18 h pulsing. Data presented in Figure 12A
are means derived from experiments with B-cell
preparations from three different donors with
duplicate incubations. Data of Figure 12B are means
of duplicate incubations from one experiment out of
two with comparable results.

None of the novel anti-CD40 mAbs was able to
significantly costimulate human B-cell proliferation
in the presence of immobilized anti-IgM or in the
presence of immobilized anti-IgM and IL-2. In
contrast, anti-CD40 mAb S2C6 costimulated human B-
cell proliferation in a concentration dependent
fashion.

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Example 16
Induction of B-Cell Proliferation
Using Anti-CD40 mAbs

The mAbs tested in Example 15 were tested for
5   their ability to induce proliferation of human B
cells in the Banchereau-like Assay described above,
i.e., by presenting the anti-CD40 mAb on adherent
cells expressing FcγRII. As antibody presenting
cells, mouse 3T6 transfectant cells expressing the
10 HR allellic form of human FcγRII were used. It was
observed that anti-CD40 mAb S2C6 together with IL-4
induced substantial proliferation of tonsillar human
B cells in this system, as assessed by measurement
of [^{3}H]thymidine incorporation. Anti-CD40 mAbs 5D12,
15 3C6 or 3A8 however, did not induce proliferation of
human B cells in this culture system (data not
shown).

Example 17
Inhibition of S2C6 Stimulated B-Cell
20   ### Proliferation Using Anti-CD40 mAbs

The anti-CD40 mAbs were also tested for their
ability to inhibit the costimulation of human B-cell
proliferation by anti-CD40 mAb S2C6 using the B-cell
Proliferation Assay described above. Human
25 tonsillar B cells (4 x 10^4 per well) were cultured in
200 μl in microwells in the presence of anti-IgM
coupled to Sepharose beads (5 μg/ml) and anti-CD40
mAb S2C6 (1.25 μg/ml). Varying concentrations of
anti-CD40 mAbs 5D12, 3C6 or 3A8 were added and
30 [^{3}H]thymidine incorporation was assessed after 3
days. As a control anti-(glucocerebrosidase) mAb
8E4 was added in similar concentrations. Barneveld
et al. Eur. J. Biochem. (1983) 134:585. Data are
means ± S.D. derived from experiments with B cells

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from two different donors with duplicate
incubations.

It was found that each of the anti-CD40 mAbs
5D12, 3A8 and 3C6 could inhibit the costimulation of
5   anti-IgM induced human B-cell proliferation by mAb
S2C6 (Figure 13).   In contrast, no significant
inhibition was seen with equivalent amounts of non-
relevant mAb 8E4, directed to β-glucocerebrosidase.
Barneveld et al., supra.   Thus, it was concluded
10 that these anti-CD40 mAbs do not deliver stimulatory
signals to the proliferation of human B cells, but,
conversely, can inhibit stimulatory signals exerted
by triggering anti-CD40 with another mAb.
Therefore, these mAbs were considered to be
15 excellent tools to investigate whether signaling via
CD40 plays a role in the stimulation of human B-cell
proliferation by EL4B5 cells.

Example 18
Effects of Anti-CD40 mAbs on EL4B5-Induced
20   Human B-Cell Proliferation
The effect of anti-CD40 mAbs on EL4B5-induced
human B-cell proliferation was tested using the B-
cell Activation Assay described above.   Human
tonsillar B cells (1000 per well) were cultured
25 together with irradiated EL4B5 cells (50,000 per
well) in the presence of 5% supernatant of activated
human T cells and 5 ng/ml PMA.   Anti-CD40 mAbs 5D12,
3C6 or 3A8 were added in varying concentrations.   As
a control, mAb MOPC-141 (IgG2b) was added.   After
30 six days of culture, [^{3}H]thymidine incorporation was
assessed.

Figure 14 shows that addition of anti-CD40 mAbs
5D12, 3C6 or 3A8 resulted in a concentration-
dependent inhibition of human B-cell proliferation.

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Data are means ±S.D. derived from experiments with B
cells from four different donors with duplicate
incubations.   [^{3}H]-thymidine incorporation values
found for incubations without mAb were (means ±
5   S.D.) 10460 ± 1843 cpm, 6982 ± 1729 cpm, 4362 ± 1020
cpm and 1543 ± 3190 in the four different
experiments, respectively.   [^{3}H]-thymidine
incorporation in B cells alone amounted to 40 ± 5
cpm and in irradiated EL4B5 cells alone 31 ± 15 cpm.

10   Very potent inhibition occurred.   At
concentrations as low as 10 ng/ml each, the three
anti-CD40 mAbs 5D12, 3C6 and 3A8 inhibited human B-
cell proliferation completely.   Half-maximal
inhibition was found at about 1 ng/ml.   In contrast,
15   isotype matched IgG2b mouse myeloma protein MOPC-141
had no significant effect on [^{3}H]-thymidine
incorporation.   Similar inhibition was observed when
[^{3}H]-thymidine incorporation was assessed at day 4 of
the culture instead of day 6, thus excluding the
20   possibility that the observed effect was due to a
change in the kinetics of the proliferation under
influence of the anti-CD40 mAb (data not shown).
         For comparison, the influence of a few mAb
directed against other B-cell surface structures was
25   investigated.   Neither anti-CD20 mAb B1 or anti-B7
mAb B7-24 (the latter mAb was generated by a
procedure similar to that used for generating the
anti-CD40 mAb used in Figure 14, in concentrations
similar to those used in the experiments with the
30   anti-CD40 mAb, had any effect on EL4B5-induced human
B-cell proliferation (data not shown).   Therefore,
it may be concluded that the inhibitory effect of
anti-CD40 mAb on EL4B5-induced B-cell proliferation
is not due to masking of the B-cell surface.

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Example 19
Effects of hCD40.Hμ on EL4B5-Induced
Human B-Cell Proliferation

In order to investigate whether EL4B5 cells
5   expressed a membrane structure which binds CD40, a
fusion protein consisting of the extracellular
domain of CD40 and human IgM constant domains CH_2,
CH_3 and CH_4 (hCD40.Hμ) was used for flow
fluorocytometric analysis. Lane et al., supra.
10   Non-activated EL4B5 cells did not bind the fusion
protein. However, upon culturing EL4B5 cells
together with PMA (5 ng/ml) and 5% human T-cell
supernatant, which are the conditions needed for
activation of human B cells, a low binding of
15   hCD40.Hμ was found (data not shown). This small
shift in fluorescence was found consistently in
three independent experiments. The minimal
activation period needed for induction of the CD40
binding was 24 hours. To determine whether binding
20   of hCD40.Hμ to the EL4B5 cells would inhibit EL4B5-
induced human B-cell proliferation like anti-CD40
mAb did, the fusion protein was titrated into
cocultures of EL4B5 cells with human B cells using
the B-cell Activation Assay described above. Figure
25   15 shows that the fusion protein did indeed inhibit
$[^3H]$-thymidine incorporation in a concentration-
dependent manner and, like the anti-CD40 mAb used in
the experiments shown in Figure 14, was able to
inhibit B-cell proliferation induced by the EL4B5
30   cells completely.

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Example 20

Effects of Anti-CD40 mAbs on Human T-Cell-Induced
Antibody Production by Human B-Cells

The antibodies were also tested for their
5   capacity to inhibit immunoglobulin production by B
cells, stimulated in a contact-dependent manner with
activated T cells using the T-cell helper assay
described above. Human tonsillar B cells (10^4 /well)
were cultured together with irradiated purified T
10   cells (3000 rad, 10^5 /well) in 96-well plates, coated
with anti-CD3 mAb and with or without different
mAbs to costimulate the T cells. After 8 days of
culture the supernatants were harvested for the
determination of immunoglobulin production by the B
15   cells. Immunoglobulin production by the B cells was
assessed by the ELISA assay described above. Anti-
CD40 mAb 5D12 was added in varying concentrations
from the onset of the cultures. As a control, mAb
MOPC-141 was added. Figure 16A shows that when T
20   cells were stimulated with immobilized anti-CD3 mAb
and costimulated with soluble anti-CD2 and anti-CD28
mAbs, addition of anti-CD40 mAb 5D12 resulted in a
concentration dependent inhibition of IgG production
by human B cells. IgM production by the B cells was
25   inhibited to the same extent. Similar results were
obtained with the anti-CD40 mAbs 3C6 and 3A8 and
with the hCD40.Hμ fusion protein.

The anti-CD40 mAbs of this invention exhibited
very potent inhibition. At concentrations as low as
30   approximately 30 ng/ml, each of the three anti-CD40
mAbs gave 50% of maximal inhibition. In contrast,
the isotype-matched IgG2b mouse myeloma protein
MOPC-141 had no effect on the immunoglobulin
production.
35   The inhibitory effect by the three anti-CD40

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mAbs was not specific for the manner of activation
of the T cells providing the CD40 ligand helper
activity. Figure 16B shows that under all the T-cell
stimulation conditions (anti-CD3 alone; anti-CD3 +
5   anti-CD2; anti-CD3 + anti-CD28; and anti-CD3 + anti-
CD2 + anti-CD28), addition of the anti-CD40 mAb 5D12
results in strong inhibition of immunoglobulin
production by the human B cells. The inhibition is
comparable to the amount of inhibition with the
10   hCD40.Hμ fusion protein, known to completely block
the CD40-CD40 ligand interaction. The percentage of
inhibition varied from 40 to 70% depending on the T-
cell activation conditions. In contrast, the
isotype-matched IgG2b mouse myeloma protein MOPC-
15   141, or human IgM (as control for the hCD40.Hμ
fusion protein) had no effect on immunoglobulin
production by the human B cells.

Deposition of Cultures

The hybridomas used in the above examples were
20   deposited in and accepted by the American Type
Culture Collection (ATCC), 12301 Parklawn Drive,
Rockville, Maryland, USA, under the terms of the
Budapest Treaty. This deposition does not indicate
that these hybridomas are necessary to practice the
25   invention described above or claimed below.

Hybridoma	Deposit Date	Accession No.
B7-24	May 6, 1993	HB 11341
3C6	May 6, 1993	HB 11340
5D12	May 6, 1993	HB 11339

30   The present invention has been described with
reference to specific embodiments. However, this
application is intended to cover those changes and

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substitutions which may be made by those skilled in
the art without departing from the spirit and the
scope of the appended claims.

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<u>SEQUENCE LISTING</u>

(1) GENERAL INFORMATION:

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                Conroy, Leah B.

   (ii) TITLE OF INVENTION: A Method for Generation of Antibodies to
        Cell Surface Molecules

  (iii) NUMBER OF SEQUENCES: 8

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      (E) COUNTRY: USA

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    (v) COMPUTER READABLE FORM:

      (A) MEDIUM TYPE: Floppy disk

      (B) COMPUTER: IBM PC compatible

      (C) OPERATING SYSTEM: PC-DOS/MS-DOS

      (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

   (vi) CURRENT APPLICATION DATA:

      (A) APPLICATION NUMBER:

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      (C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

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        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 27 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGCTGCAGC ATCTGAAGCC ATGGGCC   27

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 27 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGCGGTACCT TGCTTCTGCG GACACTG   27

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 27 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCGCTGCAGC ATCTGAAGCC ATGGGCC   27

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(2) INFORMATION FOR SEQ ID NO:4:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 61 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGCGGTACC TTACTCCATG GGCATGTATT CCTCTTCCTC GTTATCAGGA AAATGCTGTT G   61

(2) INFORMATION FOR SEQ ID NO:5:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 32 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGTAGATCT GGTCTCACCT CGCCATGGTT CG   32

(2) INFORMATION FOR SEQ ID NO:6:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 34 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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GCGTGGTACC CCACACTCCT GGGTGGGTGC AGCC   34

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 32 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGTAGATCT GGTCTCACCT CGCCATGGTT CG   32

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 61 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGTGGTACC TTACTCCATG GGCATGTATT CCTCTTCCTC ATCAGTCTTG TTTGTGCCTG C   61

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IT IS CLAIMED:

1.   A method of generating an immortalized
cell line producing a monoclonal antibody specific
against a selected membrane-associated antigen,
5   comprising
producing the membrane-associated antigen on
the cell surface of insect cells,
injecting said insect cells into a host animal,
recovering from the host animal cells capable
10   of producing antibody molecules,
immortalizing such cells for growth in cell
culture,
screening the immortalized cells for production
of antibodies specific to the membrane-associated
15   antigen in a binding assay employing non-insect
cells having the membrane-associated antigen on the
surface of the non-insect cells, and
selecting those immortalized cells producing
antibodies which bind to the membrane-associated
20   antigen on the surface of said non-insect cells.

2.   The method of claim 1, wherein said
producing further comprises
inserting DNA sequence encoding the membrane-
associated antigen operatively into a baculoviral
25   vector suitable for expression of said antigen in
selected insect cells,
transfecting insect cells with the vector, and
selecting insect cells transformed with the
vector which produce the membrane-associated antigen
30   on the cell surface of the insect cells.

3.   A method of producing sera containing
antibodies specific against a selected membrane-

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associated antigen, comprising

    producing the membrane-associated antigen on
the cell surface of insect cells,

    injecting said insect cells into a host animal,
5 and

    screening sera from said animal for production
of antibodies specific to the membrane-associated
antigen in a binding assay employing non-insect
cells having the membrane-associated antigen on the
10 cell surface.

    4. A method for preventing transplant
rejection in a patient, the method comprising
administering to a patient in need of such treatment
a therapeutically effective amount of (a) a molecule
15 that binds to the B7 antigen; and (b) an
immunosuppressive agent in a pharmaceutically
acceptable excipient.

    5. A method for preventing or treating graft
versus host disease (GVHD) in a patient, the method
20 comprising administering to a patient in need of
such treatment a therapeutically effective amount of
(a) a molecule that binds to the B7 antigen; and (b)
an immunosuppressive agent in a pharmaceutically
acceptable excipient.

25   6. A method for preventing or treating
rheumatoid arthritis in a patient, the method
comprising administering to a patient in need of
such treatment a therapeutically effective amount of
(a) a molecule that binds to the B7 antigen; and (b)
30 an immunosuppressive agent in a pharmaceutically
acceptable excipient.

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7.   The method of claims 4, 5 or 6, wherein the molecule that binds to the B7 antigen is an anti-B7 antibody.

8.   The method of claims 4, 5, 6 or 7, wherein
5 the immunosuppressive agent is selected from the group consisting of cyclosporin A, FK506, rapamycin and corticosteroids.

9.   A method for preventing or treating an antibody-mediated disease in a patient, the method
10 comprising administering to a patient in need of such treatment a therapeutically effective amount of a monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the
15 CD40 antigen prevents the growth or differentiation of the B cell, in a pharmaceutically acceptable excipient.

10.   The method of claim 9, wherein the antibody-mediated disease is selected from the group
20 consisting of IgE-mediated disease, sytematic lupus erythematosus (SLE), primary biliary cirrhosis (PBC), and idiopathic thrombocytopenic purpura (ITP).

11.   The method of claims 9 or 10 wherein the
25 monoclonal antibody is selected from the group consisting of 5D12, 3A8 and 3C6.

12.   A CD40 antigen epitope immunoreactive with an anti-CD40 monoclonal antibody wherein the binding of said monoclonal antibody to a human CD40 antigen
30 located on the surface of a human B cell prevents

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the growth or differentiation of the B cell.

13.   Monoclonal antibody B7-24.

14.   A monoclonal antibody capable of binding
to a human CD40 antigen located on the surface of a
5   human B cell, wherein the binding of the antibody to
the CD40 antigen prevents the growth or
differentiation of the B cell.

15.   The monoclonal antibody of claim 14
selected from the group consisting of 5D12, 3A8 and
10   3C6.

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Fig. 1A

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EBV-transformed
human spleen cells

mRNA

Random hexamer primers
Reverse transcriptase

cDNA

CD40

BglII forward primer
KpnI reverse primer
PCR

BglII   KpnI

BglII
KpnI

pAcC8
polyhedrin
promoter

BglII
KpnI
phosphatase

BglII   KpnI

Ligate

pAcCD40

Co-transfect
sf-9 cells
with AcNPV

Isolate
recombinant
virus

promoter   Polyhedrin

KpnI

Infect sf-9 cells

BglII

Fig. 1B

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WO 94/01547

PCT/US93/06432
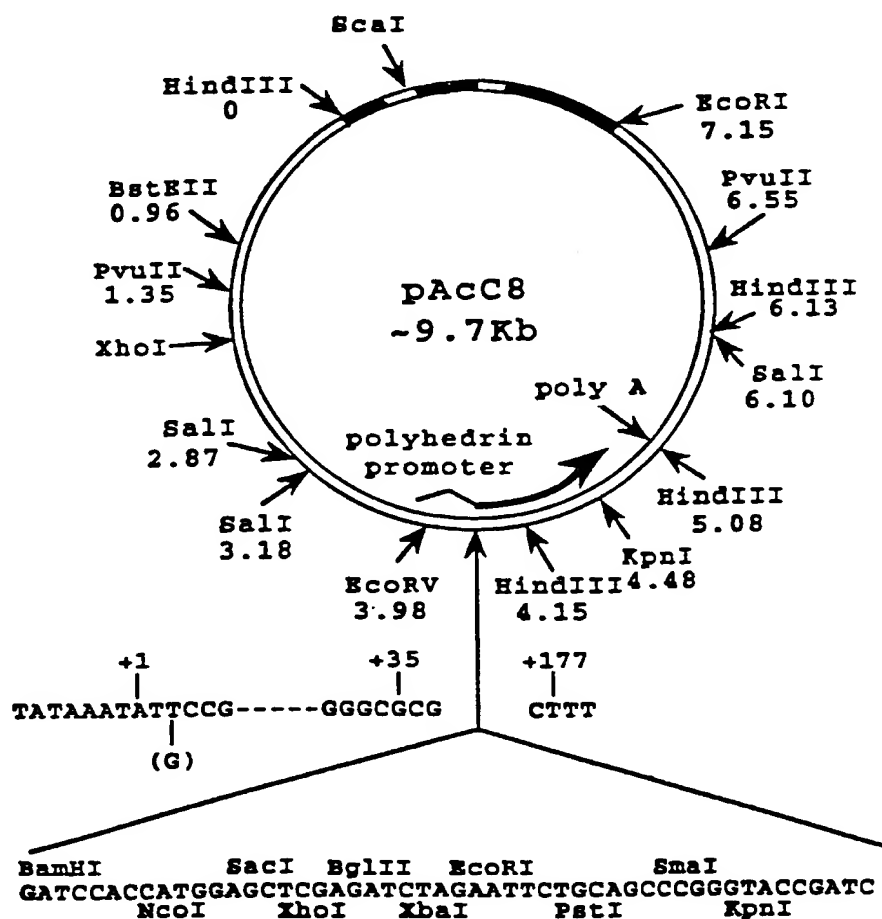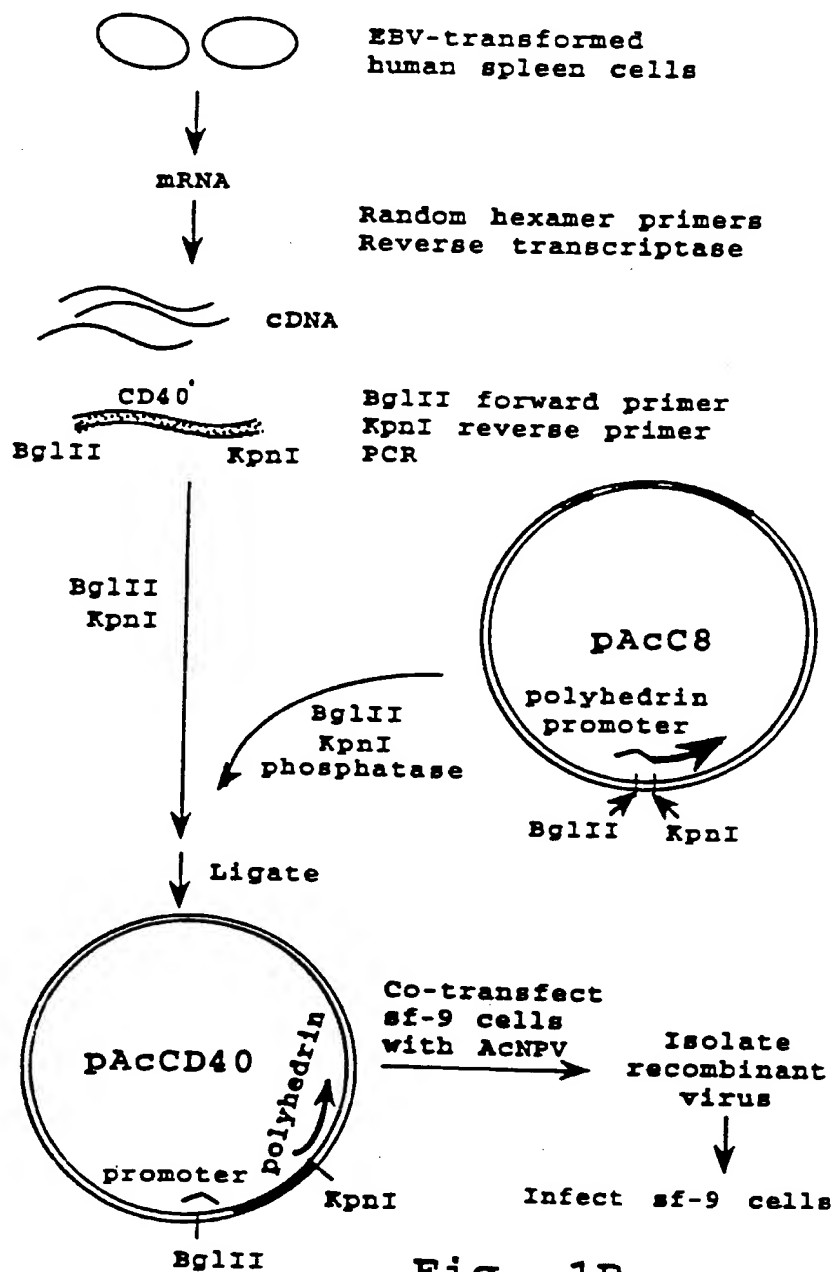
3/20

Full length B7:

Forward MR67 5'-GCG CTGCAG CATCTGAAGCCATGGGCC-3' (307-324)

Backward MR68 5'-CGC GGTACC TTGCTTCTGCGGACACTG-3' (1182-1199)

Soluble B7:

Forward MR67 5'-GCG CTGCAG CATCTGAAGCCATGGGCC-3' (307-324).

Backward MR145 5'-GCGC GGTACC TTAGCCCATGGGCATGTATCCTCTCTGCTCTTATCAGGAAAATGCTCTGTTG-3' (1022-1042)

Full length CD40:

Forward MR108 5'-GCGT AGATCT GGTCTCACCTCGCCATGGTTCG-3' (34-55)

Backward MR112 5'-GCGT GGTACC CCACACTCCTGGGTGGGTGCAGGCC-3' (882-905)

Soluble CD40:

Forward MR108 5'-GCGT AGATCT GGTCTCACCTCGCCATGGTTCG-3' (34-55)

Backward MR150 5'-GCGT GGTACC TTAGCCCATGGGCAGTATCCTCTCTCATCAGTCTCTGTTGTGCCTGC-3' (575-596)

Fig. 2

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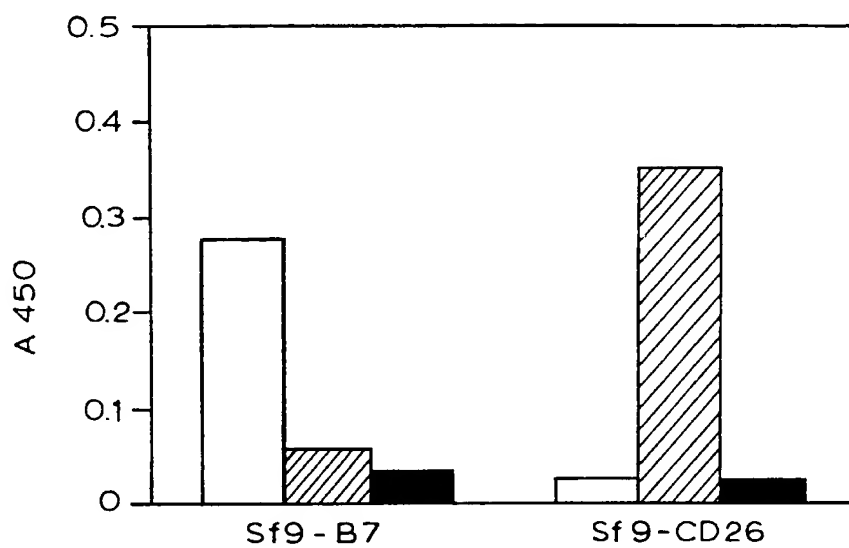
FIG. 3
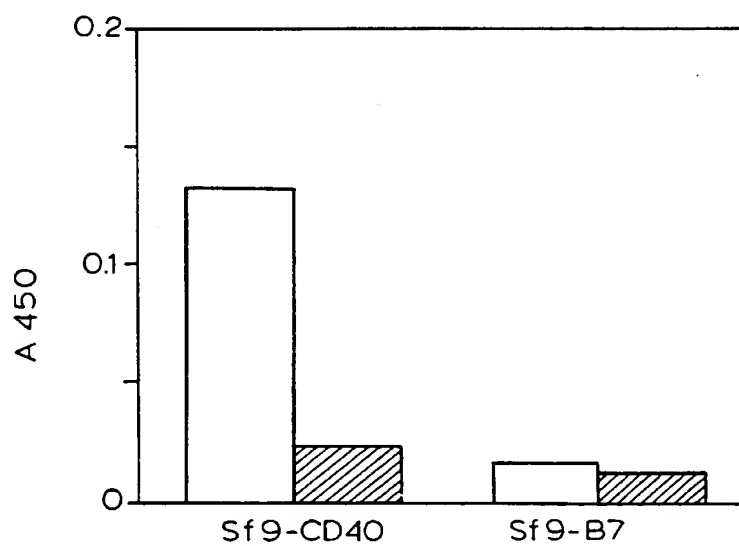
FIG. 4

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FIG. 5A
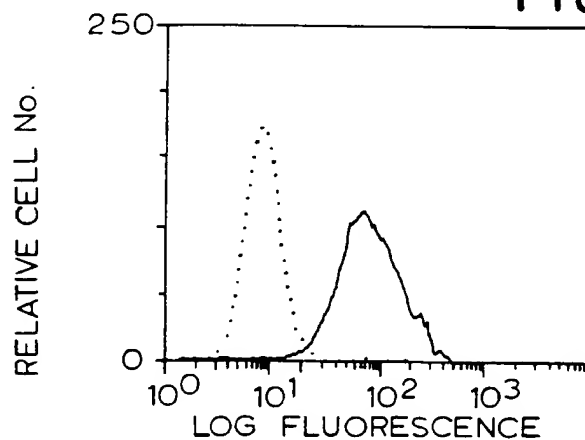
FIG. 5B
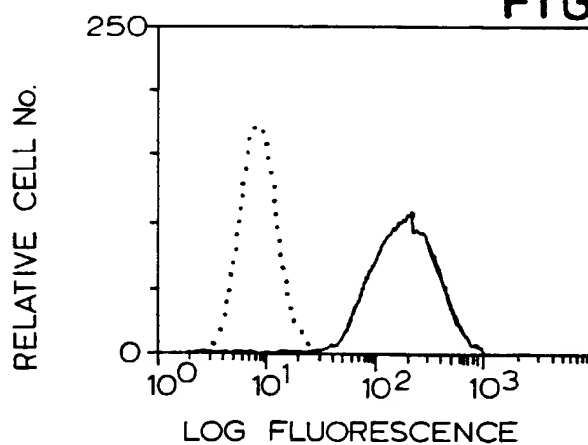
FIG. 5C

SUBSTITUTE SHEET

FIG. 6A

FIG. 6B

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FIG. 6D

FIG. 6E

FIG. 6F

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FIG. 7

Fig. 8

Fig. 9A

B7-24 (B7/BB1)

Fig. 9B

BB-1 (B7/BB1)

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Fig. 10

FIG. 11

FIG.12A

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FIG. 12B

FIG.13

FIG. 14

FIG.15

Fig. 16A

Fig. 16B